FUNGICIDE RESISTANCE AND PARASEXUAL RECOMBINATION IN *PSEUDOCERCOSPORELLA HERPOTRICHOIDES*

BY

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CONTENTS

CHAPTER 2. THE ORGANISM

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ABSTRACT

A detailed investigation of benzimidazole resistance in field isolates and laboratory mutants of *Pseudocercosporella herpotrichoides* resulted in the description of four major resistance catagories, based on the sensitivity of isolates to carbendazim: high-level (MIC = >1000 μM), intermediate-level (MIC = 50 - 200 μM), low-level (MIC = 10 - 20 μM) and sensitive (MIC = 0.6 - 2.5 μM). Cross-resistance to other benzimidazole compounds, including thiabendazole, was observed in all cases, although the level of resistance to thiabendazole was not directly related to the level of resistance to carbendazim.

Strains from each resistance catagory were tested for sensitivity to two experimental *N*-phenylcarbamate fungicides, MDPC and S32165. Wild-type carbendazim-sensitive isolates were insensitive to both compounds, as were most low-level and intermediate-level carbendazim-resistant mutants. Many high-level carbendazim-resistant strains, including most of the carbendazim-resistant field isolates showed increased sensitivity to one or both phenylcarbamates. In addition, a fifth resistance phenotype was identified, showing increased sensitivity to MDPC and S32165 but associated with only slightly reduced benzimidazole sensitivity.

Spontaneous mutants from a high-level carbendazim-resistant strain, selected for reversion to insensitivity to MDPC were readily obtained. Analysis of their fungicide response showed some to be back-mutations in which insensitivity to MDPC was associated with the loss of resistance to carbendazim, while others appeared to carry suppressor or modifier mutations producing a range of resistance phenotypes.

A study of sensitivity to a range of ergosterol biosynthesis inhibiting (EBI) fungicides revealed considerable variation

between different isolates. In general, BWR-type isolates were much less sensitive to the triazole compounds tested than BW-types, while the reverse relationship was found for the morpholine fungicide, fenpropimorph. Sensitivity to prochloraz showed much less variation. No correlation was found between benzimidazole resistance and reduced sensitivity to the EBI fungicides.

Two pathotypes of P. herpotrichoides, BW and BWR, usually distinguished on the basis of cultural morphology, are recognised. The morphological character, however, was found to be unstable, particularly in BWR-types, which readily sectored to give colonies typical of the other pathotype.

Parasexual recombination in P. herpotrichoides was demonstrated using auxotrophic mutants isolated after UV-irradiation of conidia. Unstable heterokaryons were obtained following hyphal anastomosis between complementary strains. From these heterokaryons stable prototrophs were recovered both from spores and isolated protoplasts. Genetic evidence and DNA estimations indicated that these stable prototrophs were diploids. Recombinant types were isolated from diploids after treatment with 5-fluorouracil. It was also shown that viable heterokaryons could be recovered following PEG-mediated fusion of protoplasts isolated from complementary strains.

Heterokaryon incompatibility between unrelated field isolates was tested using auxotrophic mutants obtained by selection for resistance to chlorate and selenate. Vegetative incompatibility appeared to be widespread, few positive interactions being observed, suggesting that the potential for parasexual gene exchange in the field is low. Protoplast fusion may provide a method whereby this incompatibility can be overcome in the laboratory.

To my family and friends

CHAPTER 1

GENERAL INTRODUCTION

1.1 LITERATURE REVIEW

1.1.1 Development of Fungicide Resistance

The use of chemicals to control disease imposes a strong selective pressure upon the pathogen population towards the development of resistant forms. The appearance of resistant strains depends not only on the magnitude of the selection pressure, but also on the probability of resistance to the particular control agent occurring. The latter is, to a great extent, dictated by the mode of action, or more particularly on the site specificity, of the toxicant (Wade, 1982).

Before 1968 few examples of resistance to fungicides were known, and fewer still gave cause for concern for loss of disease control in the field (Georgopoulos & Zaracovitis, 1967). The reason for this success lay in the nature of the antifungal compounds available at the time. Most were general metabolic inhibitors, affecting multiple sites within the cell, consequently multiple simultaneous mutations would have been required for resistance to arise. Of the few instances of resistance to conventional fungicides, significant problems were encountered with the appearance of resistance to the aromatic hydrocarbons and to dodine (Georgopoulos, 1977; Dekker, 1977; Szkolnik & Gilpatrick, 1969; MacNeill & Schooley, 1973; Jones & Walker, 1976; Jones, 1981; Gilpatrick & Blowers, 1974).

The introduction of specific site inhibitors, many of which were systemic in the plant and so had curative or eradicant

properties, caused a revolution in the treatment of crop diseases. Very soon after their initial use the first reports of failure of disease control, due to resistance in the pathogen population, appeared and have continued to appear regularly ever since (Delp, 1980; Dekker,1982). Indeed resistance has been reported to all the major groups of fungicide currently available (Dekker, 1977; Delp, 1980; Georgopoulos, 1982; Staub & Sozzi, 1984). Where genetic studies have been carried out resistance development to such fungicides has usually been shown to involve mutation in a single gene. Compared with resistance to the conventional fungicides, the development of resistance to site specific fungicides has often been far more dramatic. In general the mutation rate of individual loci is about 10^{-8} to 10^{-6} (Fehrmann, 1976), so that resistant individuals probably exist naturally in the population. As a result, the speed of resistance build-up can be surprisingly rapid; the foremost example of which involves the appearance of resistance to the benzimidazole fungicides.

However, the isolation of resistant types either from wild populations or laboratory strains does not imply that resistance will cause problems for disease control in the field. The selection pressure on the population, the proportion of sensitive isolates which are removed from the population as a result of using the fungicide, the stability of the resistance and the characteristics of the resistant strains will all affect the extent to which resistance becomes a practical problem. The epidemiology and life cycle of the disease agent is also of prime

significance. Pathogens in which a sexual stage is an integral part of the life cycle will have a greater potential for genetic recombination, decreasing the effects of reduced fitness by resorting resistance genes in potentially more successful combinations. Pleiotropic effects on fitness of resistance mutations will be considered later. Airborne pathogens, producing large amounts of inoculum will also show a more rapid build-up of resistant strains, and be capable of more rapid changes in population structure, than fungi in which inoculum dispersal is limited, temporally or spatially.

1.1.1.1 Development of Resistance to Benzimidazoles

Resistance to benomyl, the original benzimadazole fungicide, was first reported in Sphaerotheca fuliginia, in which it appeared within the first year of use of this compound to control powdery mildew of cucumber (Schroeder & Providenti, 1969). Rapid development of resistance also occurred in other species. Benomyl-resistant isolates of Botrytis cinerea were obtained from cyclamen which had received only three applications of the fungicide (Bollen & Scholten, 1971). Similarly widespread development of resistance in the sugar beet leafspot fungus, Cercospora beticola, was detected both in Greece and the U.S.A. within three years of the fungicide being released for use in this crop (Georgopoulos & Dovas, 1973; Ruppel, 1975). In Venturia inaequalis, resistant strains were isolated just two years after benomyl was first used on apple and resistant ascospores were even reported to have been detected the previous season (Jones, 1981).

Occasionally, benzimidazole resistance is known to have occurred in the pathogen population even before these compounds were used for their control, reflecting the natural rate of mutation to resistance (Fehrmann, 1976). Examples include Verticillium malthousei on mushroom (Wuerst & Cole, 1970, Wuerst et al, 1974) and Septoria nodorum and Pseudocercosporella herpotrichoides on wheat (Fehrmann et al, 1977; Horsten & Fehrmann, 1980a, Bateman et al, 1985). The rapid development of resistance in many of the species described above, strongly suggests that naturally occurring, resistant strains were present in these populations as well.

1.1.1.2 Development of Resistance in Pseudocercosporella herpotrichoides

The benzimidazole fungicides, carbendazim and thiophanate-methyl, were used very successfully for the control of P. herpotrichoides for almost a decade. Resistance was first seen in German isolates of the pathogen from infected wheat plants (Rashid & Schlosser, 1975). Previous studies had not revealed any resistance in a screen of more than 7×10^7 conidia, possibly because the sample was relatively small (Chidambaram & Breuhl, 1973). Further reports of the presence of resistant strains in cereal crops have since appeared, both from fields subjected to regular benzimidazole treatment and non-treated fields (Rashid & Schlosser, 1977; Fehrmann et al, 1977; Horsten & Fehrmann, 1980a; Bateman et al, 1985).

Despite the occurrence of benzimidazole resistance in other genera, no loss of eyespot control by these fungicides through

resistance was anticipated during this period. This was undoubtedly the consequence of the low frequency of resistant spores in the population (Fehrmann et al, 1982; Horsten & Fehrmann, 1980a), and the very localised spore dispersal observed in this fungus (Fehrmann & Schrodter, 1971). Indeed up to 1982 it was repeatedly concluded from field surveys and long term monitoring experiments, that there was little danger of widespread resistance in this pathogen. Up to two applications of carbendazim could be made each season without fear of build-up of any economically important resistance (Fehrmann & Weihofen, 1978; Horsten & Fehrmann, 1980a; Fehrmann et al, 1982).

However, loss of chemical control was reported in the U.K. in 1981. Subsequent investigations showed that a high proportion of isolates from the affected sites were resistant to high levels of carbendazim and several other benzimidazole fungicides (Brown et al, 1984). It was suggested that where continuous cereal production was practised regular use of carbendazim to control eyespot was likely to encourage the development of resistance in the pathogen and result in the failure of the control measures. A recent survey of winter cereal crops in England and Wales showed that 16 to 66% of the fields had resistant isolates, which accounted for between 37 and 52% of the pathogen population sampled. Economic losses due to resistance have been estimated to occur when between 7 and 30% of the fungal population is resistant (King & Griffin, 1985). Obviously therefore, the frequent occurrence of benzimidazole resistance in the P. herpotrichoides field population is a cause for concern.

1.1.1.3 Pathogenicity and Fitness

Pleiotropic reduction of pathogenicity in fungicide resistant mutants has been described in a variety of species (Fuchs & Viets-Verweij, 1975; van Tuyl, 1977a; Walmsley-Woodward *et al*, 1979; Tezuka & Ishii, 1983). Mutational resistance to the benzimidazoles is not usually associated with any alteration in pathogenicity, although some exceptions have been found (Meyer, 1976; Stover, 1977b; Georgopoulos & Dovas, 1973; Brasier & Gibbs, 1975; Ishii & Yamaguchi, 1977; Shabi & Katan, 1979; Wild, 1983). Similarly, resistance to carbendazim in *P. herpotricoides* was not associated with a reduction in pathogenicity; resistant strains were found to be as pathogenic as sensitive isolates in glasshouse tests (Horsten & Fehrmann, 1980b; Brown *et al*, 1984).

The survival of the resistant strains, in the absence of the fungicide, will obviously have an influence on the potential for failures in chemical control. Resistance to some fungicide groups is known to result in a reduction in the fitness of the fungus. Consequently the resistance allele involved may be expected to be lost from the population when the selective advantage provided by the presence of the fungicide is removed. This phenomenon has been demonstrated on a small scale, with edifenphos-resistant strains of *S. nodorum* (Horsten, 1979).

Reduced fitness, usually defined in *in vitro* tests by reduction in growth rate and sporulation, has been observed in isolates of various species resistant to the EBI's, carboximides, organophosphates or polyene antibiotics (Dekker, 1982; de Waard *et al*, 1982; Barug & Kerkenaar, 1984). Resistance to the benzimidazoles generally appears not to be linked with any

reduction in competitive ability. The existence of resistant isolates in populations, not previously exposed to these compounds, strongly suggests that these are at least as fit as sensitive forms in normal circumstances. Studies on the maintenance of resistance in populations where fungicide use has ceased, have confirmed that mutation to benomyl resistance usually has little effect on fitness (Dovas et al, 1976; Fletcher & Scholefield, 1976; McGee & Zuck, 1980; Shabi & Katan, 1980). In contrast, Ishii et al (1985) found that the proportion of high-level carbendazim-resistant isolates of Venturia nashicola in the population decreased, while the number of weakly and moderately resistant strains increased in the five years after the use of these compounds was stopped. A similar degeneration of resistance was seen with benomyl-resistant strains of Mycosphaerella fijiensis var. difformis (Stover, 1979). Thiabendazole-resistant isolates of Penicillium expansum were shown to be slower than sensitive strains to cause decay of pome fruits and sporulate, characteristics which would lead to a reduction in the proportion of resistant strains in the absence of the fungicide (Prusky et al, 1985).

Similar investigations with P. herpotrichoides have shown that in this species, fitness of benzimidazole-resistant isolates is comparable to that of sensitive strains (Horsten, 1979; Horsten & Fehrmann, 1980c; Cavelier & Leroux, 1983; Cavelier & Lepage, 1985). As a result regular use of this group of fungicides without additional measures to reduce the frequency of resistant strains in the pathogen population will inevitably lead

to further disease control failure in the future.

1.1.2 Mechanism of Resistance to Fungicides

Resistance to a particular toxicant may result from one of several mechanisms. Georgopoulos (1977) listed the major biochemical modifications leading to reduced drug sensitivity in micro-organisms:

1) inactivation of the toxicant
2) decreased conversion of non-toxic into a toxic compound
3) modification of the site of action
4) increased levels of the inhibited enzyme
5) decreased requirement for the product of the inhibited reaction
6) increased concentration of an antagonist to the toxicant
7) increased reliance on an alternative pathway
8) reduced uptake of the toxicant

Various of these mechanisms have been implicated in resistance to fungicides in fungi. Resistance to the ergosterol biosynthesis inhibiting fungicides (EBI's) for example, may involve changes in uptake associated with an inducible, active efflux mechanism, decreased conversion of the fungicide into an active form or modification of the sterol biosynthetic pathway resulting in alterations in the sterol composition of the cell membranes (Barug & Kerkenaar, 1984; Fuchs et al, 1984; Leroux & Gredt, 1984).

Resistance to the benzimidazole fungicides, in different species, has also been shown to have more than one basis. Decreased uptake of ^{14}C-labelled carbendazim by resistant cells

has been descibed in Sporobolomyces roseus (Nachmias & Barash, 1976), while in Dictyostelium discoideum, cross- resistance between acriflavine, cycloheximide and benzimidazoles was assumed to indicate altered membrane permeability to these compounds (Welker & Williams, 1983). Extracellular acid production was associated with benomyl resistance in V. malthousei; the precise mechanism of resistance was not fully clarified but resistant isolates of two other species, Sclerotinia homeocarpa and B. cinerea, did not produce the same acidification of the culture medium, suggesting that they had a different mode of resistance (Lambert & Wuerst, 1976). The main mechanism of resistance to these compounds, however, appears to involve reduced affinity for the inhibitor at the target site (Davidse 1975a).

1.1.2.1 Mode of Action of the Benzimidazole Fungicides

Early investigations of the mechanism of toxicity of this group concentrated on the inhibition of respiration, protein synthesis and DNA and RNA synthesis (Clemons & Sisler, 1971). It was soon recognised, however, that the major inhibitory effect of these compounds was on mitosis and nuclear division (Davidse, 1973; Hammerschlag & Sisler, 1973), their effect on other cellular processes being secondary. Inhibition of respiration and the differences observed between benomyl and carbendazim in some organisms, could be related to the formation of butyl isocyanate during the spontaneous breakdown of benomyl to carbendazim, which occurs in aqueous solutions (Hammerschlag & Sisler, 1972, 1973).

The similarity in the anti-mitotic effects between the benzimidazole fungicides and colchicine, a known microtubule

inhibitor, lead to the suggestion that the site of action of these compounds was the same (Davidse, 1973). That the target protein for carbendazim was indeed identical with the microtubule subunit protein, tubulin, was confirmed by a comparison of their biochemical properties and co-polymerisation with porcine brain tubulin (Davidse, 1975b). Furthermore the antitumour drug oncodazole (syn. nocodazole), known to bind to animal tubulin (de Brabander et al, 1975), was shown to have a high binding affinity for the carbendazim-binding protein from Aspergillus nidulans, and was even more fungitoxic for growth than carbendazim (Davidse & Flach, 1977). In addition binding of ^{14}C-labelled carbendazim to the fungal protein was shown to be competitively inhibited by this compound, strongly suggesting that they were both competing for the same site (Davidse & Flach, 1977).

1.1.2.2 Mechanism of Benzimidazole Resistance

Benzimidazole resistance was shown to be the result of lowered affinity of the fungal tubulin for these compounds in the resistant mutants. Mutation in the same benA gene in A. nidulans, could either cause increased binding affinity and hence increased sensitivity for carbendazim, or reduced affinity and consequently carbendazim resistance (Davidse, 1975a). Mutants resistant to thiabendazole but with negatively correlated cross-resistance to carbendazim (i.e. increased sensitivity to carbendazim) had tubulins with a correspondingly lower binding affinity for thiabendazole and increased affinity towards carbendazim. This locus was later identified as the structural gene for β-tubulin, many of the resistant mutants having electrophoretically abnormal

β-tubulins (Sheir-Neiss et al, 1978).

Reduced binding of carbendazim to β-tubulin has also been demonstrated in resistant strains of B. cinerea (Tripathi & Schlosser, 1982). Reduced uptake by resistant strains was also observed, indicating a dual mechanism of resistance in this species. In contrast, no differences in uptake or metabolism of carbendazim were found in resistant isolates of A. nidulans or V. inaequalis (Davidse & Flach, 1977; Gasztonyi & Josepovits, 1981).

The mode of action of thiabendazole appears to be the same as that of carbendazim. Tubulin from thiabendazole-resistant P. expansum isolates, with increased sensitivity to carbendazim, had a reduced binding affinity for thiabendazole and increased affinity for carbendazim (Davidse & Flach, 1978).

Alterations in the β-tubulin protein has also been shown to be the basis of benzimidazole resistance in Neurospora crassa (Orbach et al, 1986), Saccaromyces cerevisiae (Neff et al, 1983) and Physarum polycephalum (Schedl et al, 1984; Roobal et al, 1986). In N. crassa and S. cerevisiae the equivalent β-tubulin structural genes from benzimidazole-resistant and wild-type strains have been cloned and sequenced (Thomas et al, 1985; Orbach et al, 1986). In each case a single base change causing amino acid substitution in the protein product was responsible for the resistance phenotype. The high degree of homology in these β-tubulin genes allows the two species to be compared directly. Both mutational changes occur in sequences that have been highly conserved but at different positions. Unfortunately the biochemical basis for the action of many of the resistance genes that have been identified has not yet been determined.

Consequently little can be said about the diversity of resistance mechanisms in these species.

The cross-resistance to the other benzimidazole compounds regularly found in benomyl-resistant mutants, strongly suggests that they all affect the same site on the tubulin molecule. Many of the benzimidazoles used commercially as fungicides break down to yield carbendazim as the active component (Clemons & Sisler, 1969; Courtney, 1977). Thiabendazole and fuberidazole (syn. furidazole) do not form carbendazim but are fungitoxic in their own right (Erwin, 1973). This distinction may explain why the lack of cross-resistance occasionally observed between carbendazim and thiabendazole or fuberidazole (van Tuyl, 1974; Bartels-Schooley & MacNeill, 1971), has not been found between carbendazim, benomyl and the thiophanates. Mutational changes at the active site in the β-tubulin molecule may result in altered affinity for carbendazim without affecting the binding affinity for thiabendazole or fuberidazole (Davidse & Flach, 1977).

1.1.2.3 Pleiotropic Effects of Resistance Loci

Resistance mutations often produce pleiotropic effects, which may or may not affect the survival of the mutant in the field. Pleiotropic alteration of pathogenicity in such mutants has been widely studied, since it has a direct bearing on the success or failure of the chemical control measures. This aspect is discussed in section 1.1.1.3.

The most easily recognisable characters associated with resistance mutation involve changes in colony morphology and growth rate <u>in vitro</u>. Pleiotropic alteration of morphology has

been descibed in only a few instances (Beever & Bryde, 1982), but growth rate reduction has been reported to result from resistance mutations to a variety of fungicides (Bollen & Scholten, 1971; Fuchs & de Waard, 1982). Other developmental abnormalities, including altered cellular morphology and sporulation, have been described in resistance mutants (Toda et al, 1983; Welker & Williams, 1983; Ben-Yephet et al, 1975; de Waard & Gieskes, 1977; Leroux et al, 1978). Many of the benzimidazole-resistant mutants of Beauvaria bassiana isolated by Yurchenko (1979) were asporogenic and such colonies regularly formed densely sporing sectors which proved to be back-mutations to benomyl sensitivity. These non-sporing, benomyl-resistant mutants were also unable to form heterokaryons.

Mutation to fungicide resistance occasionally produces a concomitant temperature sensitivity for growth (van Tuyl, 1977b; Morris et al, 1979; Yamamoto, 1980). For instance, mutations in the imaG locus in A. nidulans, identified by van Tuyl (1977b), conferring resistance to imazalil, had several pleiotropic effects including cold-sensitivity and cross-resistance to cycloheximide, chloramphenicol and neomycin. Cold sensitivity was also found in Schizosaccharomyces pombe, associated with benzimidazole resistance (Yamamoto, 1980). Mutation at the ben-1 locus generally resulted in high-level resistance, while ben-2 or ben-3 mutants had only low-level resistance and were unable to grow in the presence of the inhibitor at 26°C. The level of resistance expressed increased with the incubation temperature, so that at 36°C these mutants were able to grow on medium

containing 10 μg carbendazim ml^{-1}.

Cold-sensitive nuclear division mutants in S. pombe were shown to be produced predominantly by mutations in one of two loci, nda-2 and nda-3. Strains with mutations in nda-3 were either resistant or supersensitive to benzimidazoles (Umesono et al, 1983). This locus was identical with ben-1 described by Yamamoto (1980), who considered it to be the structural gene for β-tubulin, by analogy with A. nidulans benA mutants, which has since been found to be the case (Hiraoka et al, 1984). Strains with mutations in the nda-2 gene were occasionally supersensitive to benomyl at the permissive temperature, 37°C (Umesono et al, 1983). The nda-2 locus has been identified as one of the two structural genes for α-tubulin in this species (Toda et al, 1983). Strains in which both nda-2 and nda-3 were present were only slightly more sensitive than the wild-type, the two loci exhibiting mutual suppression (Toda et al, 1983).

An association between temperature sensitivity and benzimidazole resistance has also been found in A. nidulans. Morris et al (1979) obtained three benA mutants resistant to benomyl and thiabendazole, which were unable to grow at 44°C. A further resistant benA mutant, benA-33, was very heat sensitive, growth of this mutant was completely inhibited at 40°C and considerably reduced at 32°C (Oakley & Morris, 1981). Heat-insensitive revertants were often found to contain extragenic suppressor mutations, two of which also suppressed fungicide resistance expression and conferred a cold sensitivity on the mutants. Of the other suppressors of heat sensitivity not affecting resistance to benomyl, one was identified as the

structural gene for α-tubulin, (tubA), (Morris et al, 1979). This mutation like nda-2 in S. pombe, confers supersensitivity to benzimidazoles when combined with the wild-type β-tubulin gene, benA (Oakley & Morris, 1981).

An explanation for the frequent association of temperature sensitivity and resistance was provided by Morris et al (1979): heat sensitivity is characteristic of temperature-dependent protein destabilisation resulting from missense mutation. Since many of the benA mutants they studied had electrophoretically abnormal β-tubulins, apparently due to missense mutations in the benA gene, the likelihood that some of these mutants would also be temperature sensitive for growth was thought to be high.

Another pleiotropic effect of resistance mutations involves cross-resistance to other fungitoxic compounds. Mutation to resistance to one benzimidazole compound usually results in cross-resistance to all the others. Exceptions do occur and have been discussed (Section 1.1.2.2). Negatively-correlated cross-resistance to other compounds in benzimidazole-resistant isolates has been described in several species. Leroux and Gredt (1979, 1983) observed that benzimidazole-resistant strains of B. cinerea and P. expansum were more sensitive to the N-phenylcarbamate herbicides propham, barban, chlorbufam and chlorpropham. A similar phenomenon has been described in P. herpotrichoides (Leroux, 1984; Leroux et al, 1985). Conversely positive cross-resistance between benzimidazole fungicides and propham has been identified in benomyl-resistant strains of V. inaequalis (Gasztonyi & Josepovits, 1981). In S. pombe, benomyl-

supersensitive nda-2 mutants, with modified α-tubulin, were more sensitive to ethyl N-phenylcarbamate (EPC) than nda-2$^+$ strains (Umesono et al, 1983).

These compounds have long been recognised to be mitotic poisons and are known to bind to plant tubulins (Ashton & Crafts, 1981; Mizuno et al, 1981). Although the actual binding site has not been identified, the studies of cross-resistance above, suggest that at least one of the sites of interference for these toxicants is at or near the benzimidazole binding site.

Several related N-phenylcarbamate compounds have been synthesised and assessed for fungitoxicity specifically towards benzimidazole-resistant strains of plant pathogens. One of these, methyl N-(3,5-dichlorophenyl)carbamate (MDPC) was much more effective against resistant isolates of B. cinerea, C. beticola, Fusarium nivale and Mycosphaerella melonis than the benzimidazole-sensitive wild-type strains (Kato et al, 1984), while in Venturia nashicola and V. pirina only those isolates with the highest levels of benomyl resistance showed increased sensitivity to MDPC (Ishii et al, 1984). Benzimidazole-resistant field isolates of P. herpotrichoides have also been shown to be extra-sensitive to MDPC (Nathaniels et al, 1985).

Cross-resistance to another mitotic inhibitor, p-fluorophenylalanine (FPA), has also been shown to occur in some A. nidulans mutants (Morris & Oakley, 1979). In this instance benomyl resistance, heat sensitivity and FPA resistance were all pleiotropic effects of the same mutation in the benA locus.

Mutational changes in membrane permeability often result in altered sensitivity to a group of toxicants and may form the

basis of resistance to many antifungal compounds (Georgopoulos, 1977). Uptake mutations, by their very nature, frequently exhibit pleiotropic cross-resistance to unrelated compounds. This feature was used to distinguish benomyl-resistant mutants of D. discoideum with altered uptake mechanisms of resistance from those resulting from mutations specifically affecting microtubule function (Welker & Williams, 1983). Permeability mutants, resistant to benzimidazoles, exhibited cross-resistance to acriflavine and cycloheximide which do not affect tubulin.

1.1.2.4 Physiological Effects of the Benzimidazoles

Besides inhibiting nuclear division and movement by disrupting the process of microtubule assembly (Oakley & Morris, 1980), benzimidazole compounds have other effects on fungi, which may indicate subsidiary sites of action. The cytokinin-like properties of benomyl in plants is well documented (Beckerson & Ormrod, 1986). In fungi low concentrations of these compounds are frequently found to stimulate mycelial growth (Oros, 1981). The basis of this growth stimulation is not understood, it may be the result of a direct effect of these compounds on growth regulation or simply a general effect on metabolism.

Enhancement of sporulation by carbendazim has been found in P. herpotrichoides (Horsten, 1979). Some resistant isolates produced significantly more conidia on agar, in the presence of this fungicide than in its absence. Similar findings were reported for some benomyl-resistant strains of Botryotinia fuckeliana (Polach & Molin, 1975).

1.1.3 Genetic Control of Fungicide Resistance

1.1.3.1 Genetic Analysis in Fungi

An understanding of the genetic basis of resistance is obviously essential for the prediction and avoidance of potential disease control loss. Genetic studies in phytopathogenic fungi have often been seriously impeded by the poor suitability of the pathogen for traditional genetic analysis, since many are totally or predominantly asexual and many important sexual species are obligate biotrophs. Consequently most genetic studies have concentrated on fungi in which sexual genetic recombination is readily manipulated *in vitro*, amongst which the non-phytopathogenic species *N. crassa*, *A. nidulans* and *S. cerevisiae* are the best characterised. Plant pathogenic species which have received most interest from geneticists include *Nectria haematococca*, *Ustilago maydis* and *V. inaequalis*. Species able to cause plant disease, in which genes for resistance to fungicides have been identified are listed in Table 1.1.

Genetic analysis using the parasexual cycle, first demonstrated by Pontecorvo *et al*, 1953; 1958) has made possible the study of the inheritance of various characters in asexual fungi (Caten, 1981). The parasexual cycle, involving mitotic segregation, has been manipulated in several species to study the inheritance of resistance to antifungal agents; particularly in *A. nidulans*, where it has often been used in conjunction with conventional sexual genetic techniques (van Tuyl, 1975; 1977a).

Table 1.1 Genetic studies in fungi: plant pathogenic species in which genes for resistance to fungicides have been studied.

ORGANISM	ANTIFUNGAL COMPOUND	REFERENCE
Oomycetes		
Bremia lactucae	metalaxyl (acylalanines)	Crute *et al*, 1985a,b
Phytophthora drechsleri	p-fluorophenylalanine	Khaki & Shaw, 1974
	chloramphenicol (antibiotics)	Khaki & Shaw, 1974
	streptomycin (antibiotics)	Khaki & Shaw, 1974
P. capsici	streptomycin (antibiotics)	Timmer *et al*, 1970
P. cactorum	streptomycin (antibiotics)	Shaw & Elliott, 1968
Ascomycetes		
Cochliobolus carbonum	cycloheximide (antibiotics)	MacKenzie *et al*, 1971 a,b
	cadminate (cadmium succinate)	Mackenzie *et al*, 1971 a,b
C. heterostrophus	cycloheximide (antibiotics)	Fry *et al*, 1984 Leach *et al*, 1982
	ethionine (*S*-ethyl-L-homocysteine)	Leach *et al*, 1982
C. sativus	anisomycin (antibiotics)	Tinline, 1961
Ceratocystis ulmi	carbendazim (benzimidazoles)	Brazier & Gibbs, 1975 Webber *et al*, 1986

Table 1.1 continued

ORGANISM	ANTIFUNGAL COMPOUND	REFERENCE
Mycosphaerella fijienis var. *difformis*	benomyl (benzimidazoles)	Stover, 1977a,b Stover, 1979
Pyricularia oryzae	kasugamycin (antibiotics)	Taga *et al*, 1979 Uesugi & Katagiri, 1977
Nectria haematococca	PCNB, TCNB (aromatic hydrocarbons)	Georgopoulos, 1970 Georgopoulos & Panopoulos, 1966
	dodine	Kappas & Georgopoulos, 1970
Venturia inequalis	antimycin A (antibiotics)	Leben *etal*, 1955
	dodine	Yoder & Klos, 1972 Polach, 1973
	benzimidazoles	Jones & Ehret, 1976 Kiebacher & Hoffman, 1981
	benomyl (benzimidazoles)	Martin *et al*, 1981 Shaki *et al*, 1983 Katan *et al*, 1983 Stanis *et al*, 1983
V. nashicola	benomyl, thiophanate-methyl (benzimidazoles)	Ishii & Yanase, 1983 Ishii *et al*, 1984
V. pirina	carbendazim (benzimidazoles)	Shabi & Katan, 1979
	benomyl (benzimidazoles)	Shabi *et al*, 1986
Basidiomycetes		
Ustilago hordei	carboxin (carboxamides)	Ben-Yephet *et al*, 1974, 1975
	benomyl (benzimidazoles)	Ben-Yephet *et al*, 1974, 1975

Table 1.1 continued

ORGANISM	ANTIFUNGAL COMPOUND	REFERENCE
U. maydis	carboxin (carboxylamides)	Georgopoulos & Sisler, 1970 Georgopoulos & Ziogas, 1977 Georgopoulos *et al* 1972
	chloroneb (aromatic hydrocarbons)	Tillman & Sisler, 1973 van Tuyl, 1977a
	benomyl (benzimidazoles)	van Tuyl, 1977a
U. violacea	chloroneb, botran (aromatic hydrocrbons)	Garber *et al*, 1982
	cycloheximide (antibiotics)	Garber *et al*, 1982
	carboxin (carboxamides)	Garber *et al*, 1982
	thiabendazole (benzimidazoles)	Garber *et al*, 1982
Deuteromycetes		
Aspergillus niger	chloroneb (aromatic hydrocarbons)	van Tuyl, 1977a
	cycloheximide (antibiotics)	van Tuyl, 1977a
	oligomycin (antibiotics)	van Tuyl, 1977a
	acriflavine (antibiotics)	van Tuyl, 1977a
	pimaricin (antibiotics)	van Tuyl, 1977a
	benomyl (benzimidazoles)	van Tuyl, 1977a
	imazalil (imidazoles)	van Tuyl, 1977a

Table 1.1 continued

ORGANISM	ANTIFUNGAL COMPOUND	REFERENCE
Cladosporium cucumerinum	carboxin (carboxamides)	van Tuyl, 1977a
	benomyl (benzimidazoles)	van Tuyl, 1977a
Fusarium oxysporum	benomyl (benzimidazoles)	Molnar *et al*, 1986
Penicillium expansum	o-phenylphenate (aromatic hydrocarbons)	Beraha & Garber, 1966
P. italicum	o-phenylphenate (aromatic hydrocarbons)	Beraha & Garber, 1980
	thiabendazole (benzimidazoles)	Beraha & Garber, 1980

However, the number of genera in which parasexual genetic analysis has been applied is still small and includes few important plant pathogenic species. As a result our knowledge of the genetic basis of resistance has been derived from a few, genetically well-characterised fungi.

1.1.3.2 Genetic Basis of Resistance

Acquired resistance to antifungal compounds that is stably transmitted from one generation to the next, and maintained even in the absence of the fungicide must be genetic in origin, arising by mutation. Unstable resistance of a non-mutational nature does, however, occur and may be responsible for quite high levels of resistance (Parry & Wood, 1959a,b). Georgopoulos (1982) termed this "Phenotypic Resistance". Phenotypic fungicide

resistance can be induced *in vitro* in naturally sensitive strains by culturing on successively greater concentrations of the inhibitory compound.

MacNeill and Schooley (1973) produced phenotypically resistant strains of *V. inaequalis* with levels of resistance to dodine comparable to those of strains with mutational resistance. In addition the level of resistance of one isolate exhibiting stable genetic resistance was further increased by continuous culturing in the presence of dodine until inhibition of growth by the fungicide was no longer detectable. In all instances this "transient" resistance was lost when strains were cultured briefly on medium lacking the fungicide.

Phenotypic resistance is commonly observed in fungi exposed to the group of fungicides collectively known as the ergosterol biosynthesis inhibitors (EBI's). Fuchs and Viets-Verweij (1975) induced phenotypic resistance to triforine in the phytopathogenic fungi *Ascochyta pisi*, *Ceratocystis fimbriata*, *C. ulmi*, *Cladosporium cucumerinum*, *Verticillium albo-atrum* and *V. dahliae*. Adapted strains were able to grow on medium containing up to 400 µg triforine ml^{-1} and the resistance was lost after culturing on triforine -free medium. Phenotypically resistant strains of *C. cucumerinum* showed a degree of cross-resistance to triarimol, though only at a low level.

Attempts to induce phenotypic resistance to benzimidazoles have invariably failed (Ben-Yephet *et al*, 1974; Meyer, 1976). Duinveld and Beijersbergen (1977) obtained highly benomyl-resistant strains of *Fusarium oxysporum* f.sp. *tulipae*, a relatively insensitive species (ED_{50} = 3-8 µg ml^{-1}) by

transferring mycelium to medium containing successively higher concentrations of benomyl. The resistance obtained was stable, did not show any reduction after cultivation for one year on benomyl-free medium and was presumably due to a mutational change in sensitivity.

1.1.3.2.1 Extrachromosomal Control

Extrachromosomal genes for resistance to various drugs are well known in bacteria, situated on plasmids transmissible between individuals of the same or unrelated species. Several species of plant pathogenic fungi have been shown to contain plasmid- or virus-like particles (Hollings, 1982), which, at least in some cases, appear to be important in the expression of pathogenicity and host specificity, usually inducing hypovirulence (Hollings, 1978; Hashiba *et al*, 1984). Evidence for the involvement of plasmids in resistance to antifungal compounds in mycelial fungi is scarce, although a DNA plasmid in *S. cerevisiae* has been found to be associated with resistance to oligomycin (Guerineau *et al*, 1974).

Fungi also contain mitochondrial DNA, which has been shown to be responsible for some cases of resistance to compounds inhibitory to mitochondrial processes. For example, resistance to triethyltin and other organotin compounds, which inhibit oxidative phosphorylation in *S. cerevisiae*, has been shown to be under extrachromosomal control (Lancashire & Griffiths, 1971). Extrachromosomally inherited resistance to oligomycin, which also inhibits phosphorylation, is controlled by two genes in this species, as is resistance to chloramphenicol, a mitochondrial

protein synthesis inhibitor (Georgopoulos, 1977). Similarly chloramphenicol resistance was caused by extrachromosomal mutation in the wood-rotting basidiomycete, Sistotrema brinkmannii (Anderson & Cenedese, 1984), while resistance to oligomycin or chloramphenicol in A. nidulans can result from mutation in an extranuclear genetic element (Rowlands & Turner, 1973).

Not all cases of resistance to inhibitors of mitochondrial function however, are due to extrachromosomal mutations, as examples of nuclear genes for both oligomycin and chloramphenicol resistance exist. Often such mutations result in a broader pattern of resistance, conferring cross-resistance to compounds of similar or even apparently unrelated mode of action. A nuclear mutation for oligomycin in S. cerevisiae, for example, confers cross-resistance to eleven other mitochondrial inhibitors including chloramphenicol, antimycin A and triethyltin bromide (Rank & Beck-Hansen, 1973).

Only one instance of apparent extranuclear inheritance of benzimidazole resistance has been reported (Stover, 1977a). Benomyl "tolerant" strains of M. fijiensis var. difformis, causing black sigatoka disease of banana, were isolated from lesions from plantations which had been sprayed with benomyl or thiophanate-methyl regularly for 18 months. Resistant strains were morphologically distinct, having an increased growth rate in culture, 4 to 6 times that of the wild-type sensitive isolates, and were avirulent. The resistance to benomyl was not transmitted either to conidia or ascospores, only being observed in hyphal

isolates. The resistant strains were apparently stable in culture and no sectors were formed. Stover (1977a) suggested that the basis of this resistance was an extranuclear inherited factor. Subsequently similar fast-growing, avirulent, benomyl-resistant isolates have been obtained from lesions caused by M. musicola, a closely related species, from other areas of Central America (Stover, 1977c).

1.1.3.2.2 Nuclear Control

Most genetic studies in fungi have revealed a chromosomal basis for fungicide resistance. In many cases resistance has been shown to be the result of a single mutational event and its inheritance is typical of a single Mendelian gene (van Tuyl, 1977a). The number of genes that may be involved in the control of resistance depends, to a large extent, on the possible mechanisms of resistance. Resistance mechanisms which involve modification of the site of action are usually controlled by one or a few genes, as with carboxamide or benzimidazole resistance. Resistance which results from alteration in uptake or in the metabolism of the compound, frequently exhibits multigenic control, since many genes will affect either membrane permeability or the metabolic pathways involved in toxification or detoxification of the fungicide. Uptake mutants causing reduced sensitivity to p-fluorophenylalanine (FPA) have been studied in A. nidulans (Srivastava & Sinha, 1975). Ten loci have been identified, some altering the activity of a permease which concentrates FPA in the mycelium, others affecting regulatory enzymes responsible for the production of the permease proteins.

Similarly, Jund and Lacroute (1970) identified seven genes controlling resistance to 5-fluoropyrimidines in S. cerevisiae, two of which were shown to be associated with loss of specific permease activity. Mutations at the other loci affected the regulation of the pyrimidine pathway, and consequently the metabolism of the inhibitors.

Multigenic resistance has been observed with other fungicide groups. Resistance to the ergosterol biosynthesis inhibitor imazalil, in A. nidulans, is based on a multigenic system of eight loci (van Tuyl, 1977b). Genetic studies of resistance to other members of this group have not been reported but it seems highly probable that similar multiple gene systems are present, in view of the variation in resistance level and patterns of cross-resistance observed (Fuchs & de Waard, 1982; Barug & Kerkenaar, 1984).

Dodine resistance in N. haematococca is based on at least four major genes, two of which have been shown to be influenced by modifiers (Kappas & Georgopoulos, 1970). In V. inaequalis two or more genes are known to be involved in resistance to this compound (Yoder & Klos, 1972; Polach, 1973). Although the genetic basis of resistance has been elucidated and it is known that dodine affects the permeability of the plasma membrane (Kaars Sijpesteijn, 1982), the physiological mechanism of this resistance is not known.

The number of loci found to confer resistance to a fungicide group is often correlated with the number of mutants studied, since a larger sample of resistant strains increases the chances of isolating those mutations occurring at low

frequencies. Georgopoulos (1963) analysed twelve mutants of N. haematococca exhibiting resistance to the aromatic hydrocarbons PCNB and TCNB and was able to assign them to three, freely segregating loci. When a further one hundred mutants were studied two more loci were recognised (Georgopoulos & Panopoulos, 1966).

Resistance resulting from a change in the site of action of the toxicant cannot only be caused by different alleles but also by different genes, since the products of a number of loci can contribute to site modification. Such a phenomenon has been observed in carboxamide-resistant mutants of U. maydis. Georgopoulos and Sisler (1970) described two types of mutation leading to alteration in the sensitivity of succinate dehydrogenation, the site of inhibition by fungicides in this group (Ulrich & Mathre, 1972), to carboxin. The mutations could be distinguished by their differential effects on antimycin A resistance, which inhibits a separate site in the electron transport system. One mutant had greatly increased sensitivity to this antibiotic while the other had no pleiotropic effect. In A. nidulans three unlinked genes each reduced inhibition of three enzymes involved in succinate dehydrogenation caused by carboxin (Gunatilleke et al, 1976). None of the carboxin resistance mutations in U. maydis or A. nidulans has been found to alter uptake of carboxin through the plasma membrane (Georgopoulos et al , 1972; Gunatilleke et al, 1976).

1.1.3.2.3 Genetic Basis of Benzimidazole Resistance

Since the first genetic studies of resistance in A. nidulans (Hastie & Georgopoulos, 1971), many reports on the inheritance of benzimidazole resistance have appeared. In most cases a monogenic basis for resistance was found (Table 1.2), usually conferring a high level of resistance to a range of benzimidazole fungicides. Hastie and Georgopoulos (1971) identified two loci in A. nidulans, one conferring a high level of resistance (ben-1) and the other a lower level (ben-2). Van Tuyl (1975, 1977a) was able to identify three non-allelic resistance loci in A. nidulans, again differing in the level of resistance conferred; one (benA) producing mutants with a high level of resistance and the other two (benB, benC) giving lower levels of resistance. benA was found to be allelic with the ben-1 gene of Hastie and Georgopoulos, and it was suggested from a comparison of their linkage map positions, that benB was allelic with ben-2 (van Tuyl, 1977a). Lacava (1979) also identified a single gene for benomyl resistance (ben-1) in A. nidulans, located at the same position as benA of van Tuyl, and presumably allelic to it. This gene has been shown to be one of two structural genes for β-tublin in A. nidulans, which code for two electrophoretically different β-tubulin proteins (β_1 & β_2). The other (tubC) codes for a species of tubulin apparently expressed only during conidiation (β_3). Mutation in benA produces strains resistant to benzimidazoles for vegetative growth, but which are unable to sporulate in the presence of these compounds, while additional mutation in the tubC gene restores conidiation under

these conditions (Sheir-Neiss et al, 1978; May et al, 1985; Morris, 1986). Altered sensitivity to benzimidazoles in A. nidulans could also result from mutation in the structural gene for α-tubulin (tubA). Strains carrying the tubA-1 mutation were extra-sensitive to benomyl (Morris et al, 1979). The function of benB and benC genes are as yet unknown, but do not appear to code for tubulin proteins. Furthermore the influence of a second α-tubulin structural gene (tubB) on benzimidazole sensitivity expression has yet to be determined (Morris, 1986).

Table 1.2 Numbers of genes for benzimidazole resistance identified in different species.

SPECIES	NO. LOCI	REFERENCE
Aspergillus nidulans	3	Hastie & Georgopoulos, 1971 van Tuyl, 1977a Lacava, 1979
A. niger	1	van Tuyl, 1977a
Ceratocystis ulmi	1	Brasier & Gibbs, 1975 Webber et al, 1986
Dictyostelium discoideum	>5	Welker & Williams, 1983
Fusarium oxysporum	2	Molnar et al, 1986
Mycosphaerella fijiensis var. difformis	1 extrachromosomal	Stover, 1979 Stover, 1977a
Neurospora crassa	1	Borck & Braymer, 1974
Penicillium italicum	2-3	Beraha & Garber, 1980

Table 1.2 continued

SPECIES	NO. LOCI	REFERENCE
Physarum polycephalum	4	Burland et al, 1984 Schedl et al, 1984
Saccharomyces cerevisiae	1	Neff et al, 1983 Thomas et al, 1985
Schizosaccharomyces pombe	5	Yamamoto, 1980 Umesono et al, 1983 Roy & Fantes, 1983 Hiraoka et al, 1984 Toda et al, 1984
Talaromyces flavus	1	Katan et al, 1984
Ustilago hordei	polygenic	Ben-Yephet, 1974, 1975
U. maydis	1	van Tuyl, 1977a
U. violacea	1	Garber et al, 1982
Venturia inaequalis	1	Jones & Ehret, 1976 Kiebacher & Hoffmann, 1981 Martin et al, 1981 Shabi et al, 1983 Katan et al, 1983 Stanis & Jones, 1984
V. nashicola	1	Ishii & Yanase, 1983 Ishii et al, 1984
V. pirina	1	Shabi & Katan, 1979 Shabi et al, 1986

Evidence for the potential involvement of a number of loci in benzimidazole resistance was strengthened by the study of resistance in S. pombe (Yamamoto, 1980). One gene (ben-1) conferred high-level resistance to both carbendazim and thiabendazole and the other two (ben-2, ben-3) a lower level of

resistance. ben-1 is identical with nda-3, the structural gene for β-tubulin in this yeast (Umesono et al, 1983; Hiraoka et al, 1984). Two genes for α-tubulins occur in S. pombe, one (nda-2) has been shown to cause increased sensitivity to benzimidazoles and is essential for growth. The other, like tubB in A. nidulans is non-essential and its effect on benzimidazole sensitivity unknown (Umesono et al, 1983; Toda et al 1984; Morris, 1986). Neither of the two low-level resistance genes, ben-2 and ben-3, have been shown to be tubulin genes. Their homology to benB and benC of A. nidulans remains uncertain, however, low-level resistance expression in S. pombe appears to be temperature dependant, a characteristic which does not seem to have been described in A. nidulans (Yamamoto, 1980). An additional benzimidazole resistance gene (ben-4) in S. pombe was descibed by Roy and Fantes (1983), which was not allelic with either nda-2, nda-3, ben-2 or ben-3, or with the stuctural gene for the α_2-protein (Toda et al, 1984).

In the slime mould, Physarum polycephalum, four resistance loci have been identified, benA to benD (Burland et al, 1984). benA and benC mutations confer benzimidazole resistance only to the myxamoebic phase of this organism, while mutations in benB and benD result in resistance in both mxyamoebae and plasmodia. Schedl et al (1984) showed that benD mutations were allelic to the betB locus, a structural gene for β-tubulin, one of at least three which have been found. The β-tubulin encoded by this gene (β_1-B-tubulin), occurs in both myxamoebae and plasmodia. An electrophoretically identical β-tubulin (β_1-A-tubulin), produced

only in myxamoebae, is coded for by the betA locus. This gene was closely linked to the benA resistance locus and is probably allelic to it. Mutation in either betA (benA) or betB (benD) results in benzimidazole resistance in myxamoabae, showing that the resistance is epistatic.

The resistance locus benC was shown to be linked to the α-tubulin structural gene altC, and may be allelic to it. In which case the altC gene product must be either specific to the myxamoebic phase, since benC only confers resistance to benzimidazole compounds to myxamoebae, or provide only a small proportion of the α-tubulin present in plasmodia, so that resistance is not expressed (Roobal et al, 1984). The fourth resistance gene, benB, was not linked with any of the known α- or β-tubulin genes, and its mode of action remains unclear. In addition the function of the gene products of the remaining three α-tubulin genes and at least one β-tubulin gene that have been identified and their role, if any, in the expression of resistance to benzimidazoles has yet to be determined.

A similar model, involving the expression of two separate β-tubulin genes, was proposed by Molnar et al (1986) to explain the observed synergy between the two benzimidazole resistance genes in Fusarium oxysporum. If each of these genes coded a different β-tubulin protein, either of which could support normal growth, mutation in one could lead to a moderate level of resistance to benzimidazole fungicides, since a proportion of the β-tubulin would be benzimidazole-sensitive. The presence of mutant alleles at both loci in the same cell, would then account for the increased level of resistance seen in the double mutants.

This hypothesis awaits confirmation by biochemical studies.

Beraha and Garber (1980) used the parasexual cycle to study the inheritance of thiabendazole resistance in Penicillium italicum. They produced evidence for the presence of either two or three closely linked genes, conferring different levels of resistance.

Polygenic inheritance appears to be operating in Ustilago hordei (Ben-Yephet et al, 1975). However, genetic analysis was not straight forward, as the mutation to resistance was associated with a reduction in the proportion of teliospores germinating to produce sporidia. Carboxin resistance also appeared to be under polygenic control in this species (Ben-Yephet et al, 1975).

Ascospore-transmitted resistance to benomyl in M. fijiensis var. difformis was recognised by Stover (1977b). Previously only hyphal resistance had been recognised and thought to be of extranuclear genetic origin (Stover, 1977a). Strains carrying the resistance mutation were able to grow on medium containing 200 µg benomyl ml^{-1}. The level of resistance shown by field isolates, and also their frequency in the population decreased, however, once application of benomyl in the plantations ceased. This indicated that the resistance itself was unstable and that resistant strains competed poorly with sensitive isolates in the absence of a strong selective pressure (Stover, 1977b, 1979). Analysis of the resistance of ascospores from single asci indicated that a single nuclear gene was involved (Stover, 1979).

1.1.3.3 Mutation Frequency

The frequency of spontaneous mutation will depend on the number of loci at which mutation can produce a resistant phenotype and on the mutability of the individual loci. Resistance which may arise as a change in one of many genes will show a characterisically high mutation frequency. The rate of spontaneous mutation to FPA resistance in conidia of A. nidulans for instance, was calculated to be 2.6×10^{-3} (i.e. 1 in 3.8×10^{2} conidia) (Chatoo & Sinha, 1974). Spontaneous mutation frequencies for benzimidazole resistance have been reported for several fungal species (Table 1.3). Most estimates fall within the range 1×10^{-6} to 1×10^{-7}. Spontaneous mutation frequencies to carbendazim resistance in P. herpotrichoides however, are somewhat lower than those reported for many of the other species.

Mutation rate in Septoria nodorum appears to depend on the level of resistance selected. Low-level resistance (MIC = 5 µg carbendazim ml^{-1}) occurring at a higher frequency than high-level resistance (no inhibition by 5 µg carbendazim ml^{-1}). These findings have obvious implications for the choice of fungicide concentration when screening for resistance (Horsten, 1979; Horsten & Fehrmann, 1980a).

1.2 Objectives

For reasons that have been described, economic crop loss resulting from the development of benzimidazole resistance in P. herpotrichoides was largely unexpected (Trow-Smith, 1982). This feature, together with the current confusion over the status of

Table 1.3 Spontaneous mutation frequencies for resistance to benzimidazole fungicides in fungi

SPECIES		MUTATION FREQUENCY	REFERENCE
Aspergillus nidulans		3×10^{-6}	Lacava (1979)
Colletotrichum lindemuthianum		1×10^{-6} - 1×10^{-7}	Meyer (1976)
Beauvaria bassiana		1×10^{-6}	Yurchenko (1979)
Ceratocystis ulmi		7×10^{-9}	Brasier & Gibbs (1975)
Fusarium oxysporum f.sp. *melonis*		1.1×10^{-8}	Bartels-Schooley & MacNeill (1971)
Pseudocercosporella herpotrichoides		1×10^{-7} - 4.2×10^{-10}	Fehrmann et al (1977, 1982) Fehrmann & Weihoffen (1980a) Horsten & Fehrmann (1980a)
Septoria nodorum	low-level	3.6×10^{-7}	Horsten & Fehrmann (1980a)
	high-level	4.5×10^{-9}	

the two major pathotypes within the population, suggested that futher analysis of the problem, using where possible a genetic approach, was required. Consequently the main aims of the present work were:

1) to characterise the expression of resistance to the benzimidazole fungicides in *P. herpotrichoides*, using both field isolates and laboratory mutants,

2) to develop a system for genetic study in this pathogen, based on parasexuality.

CHAPTER 2

THE ORGANISM

In view of the interest in the structure of, and changes in, the field population of the eyespot fungus, it is perhaps pertinent to devote some time to a description of the taxonomic status and epidemiology of the pathogen, and its behaviour in culture.

2.1 MATERIALS AND METHODS

2.1.1 Media

The main solid medium used for the in vitro culture of P. herpotrichoides was malt extract-yeast extract-glucose agar (MYG; g l^{-1}, malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 20). Other complete media used included malt extract-glucose agar (MA; g l^{-1}, malt extract, 10; glucose, 10), and potato-dextrose agar (PDA). A defined medium, used for the identification of auxotrophic mutants and for selection of "hybrid" colonies, was a modified form of those described by Macer (1961a) and Deacon (1973 b) and contained (g l^{-1}) K_2HPO_4, 0.1; $NaNO_3$, 0.2; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; NaCl, 0.5; trace elements, 1 ml (Macer, 1961a); thiamin HCl, 0.1mg; glucose, 10; agar (Difco-Bacto), 20.

A nutrient-deficient medium, required for the production of conidia, consisted simply of water agar (TWA; agar, 20g l^{-1} dissolved in tap water). All media were sterilized by autoclaving at 121 ^{o}C for 30 min.

2.1.2 Strain Isolation

P. herpotrichoides was isolated from infected wheat or barley straws as follows. Pieces of straw 1 to 2 cm long were surface sterilized by immersion in 25% chloros for 30 s and plated onto MYG agar containing 100 µg ml^{-1} streptomycin sulphate and 50 µg ml^{-1} erythromycin or ampicillin to reduce bacterial growth. P. herpotrichoides mycelium grew out onto the agar surface within 2 to 3 weeks at 19°C and was transferred to TWA medium to induce sporulation. Spores, scraped off the agar surface, were filtered through 2 to 3 layers of sterile muslin, to remove hyphal fragments, washed with sterile distilled water and plated onto MYG agar to give single colonies.

Most strains from other sources were also induced to sporulate and colonies arising from single spores were isolated. This was done to reduce the chance of mixed cultures being used in subsequent experiments and to ensure genetic uniformity, as far as was possible, within each strain.

The origin and characteristics of field and species-type isolates used, are listed in Appendix I.

2.1.3 Maintenance of Stock Cultures

Isolates were maintained in several ways. Routinely used strains were kept on MYG agar plates and sub-cultured regularly every 2 to 3 months. MYG slant cultures were not successfully used for the storage of isolates, since the fungus rapidly lost viability when stored in this way. For long-term maintenance of field isolates and mutants, cultures were kept on straw. Pieces of internodal, wheat straw, approximately 2 to 3 cm long were

sterilized by autoclaving for 30 min at 121°C in glass 1 oz bottles containing 3 ml of distilled water. After inoculation, the fungus was allowed to colonise the straw segments for 1 to 2 months at 19°C. Straw stock cultures were then kept at 4°C until required. Isolates maintained in this way, remained viable for over 2 years without apparent loss of pathogenicity. Sporulating cultures, growing on TWA slants, could be stored at 4°C. Viability under these conditions was maintained for at least 6 months.

2.1.4 Spore Production

Conidia were produced under nutrient-deficient conditions, on TWA plates at 19°C; the time required depended on the strain of the fungus and the inoculum density. When larger numbers of spores were required they were produced by a modification of the method described by Reinecke and Fokkema (1979). Plates containing MYG agar, were seeded with large numbers of spores and incubated at 19°C for 2 to 3 days. Abundant microcyclic conidia formation usually ensued, resulting in a slimy, cream-coloured layer of conidia on the surface of the agar. Conidia produced in this way could be repeatedly sub-cultured on fresh MYG agar for up to 3 cycles before reverting to the mycelial, non-sporing form. Spores were collected by suspension in sterile distilled water, and filtration through 3 layers of muslin to remove mycelial fragments. Conidia were washed twice in sterile distilled water, by centrifugation, before use.

2.1.5 Growth Studies

Growth rate studies were performed *in vitro* using either MYG agar or PDA. Colonies were grown from agar plug inocula, 3 to 6 mm in diameter, at 19°C for up to 26 days. Colony diameters, taken at right angles, were measured and used to calculate radial growth rate (mm day^{-1}). Spore production on TWA of colonies, grown from point inocula, was assessed visually with the aid of a microscope (Vickers, x10 objective) using phase contrast, and scored on a scale of increasing abundance (-, +, ++, +++). A quantitative assessment of spore production was made when cultures were grown on TWA from inoculum spread over the entire agar surface. Agar plugs (6 mm diameter) were taken from random points on the plate and vigorously shaken in 1 ml sterile distilled water. The number of spores in the resulting suspension was determined using a counting chamber. Colonies from single spores were obtained as described in Chapter 2 (Section 2.1.2).

2.1.6 Chemicals and Media Ingredients

Malt extract, yeast extract and PDA were purchased from Oxoid Ltd., Basingstoke. All other chemicals were of analytical reagent grade and either from BDH Chemicals Ltd., Poole, or Fisonsplc, Loughborough.

2.2 TAXONOMIC HISTORY OF *PSEUDOCERCOSPORELLA HERPOTRICHOIDES*

The history of the identification of the eyespot pathogen was thoroughly reviewed by Davies (1970). Only a brief description, therefore, will be given here.

The symptoms of the disease caused by Pseudocercosporella herpotrichoides (Fron) Deighton on cereals were first described in France by Pluchet in 1878. It was several decades later, however, before a formal identification was made. Fron (1912) described the fungus and provisionally named it Cercosporella herpotrichoides, since he considered it to be the perfect form of Leptosphaeria herpotrichoides. Attempts to induce the alternative form in either species were unsuccessful and led Foex (1914) to conclude that they were unrelated.

Much of the confusion about the identity of the eyespot pathogen stemmed from the difficulty of producing conidia in pure culture. Foex and Rosella (1930) isolated a sterile fungus with which they were able to induce symptoms typical of eyespot disease, in inoculated plants. Since no spores were produced they were unable to identify the pathogen and called it "Champignon x". Sprague (1931), working in the U.S.A., was the first to obtain spores in culture and so confirm that the eyespot pathogen was indeed C. herpotrichoides. Using the same technique conidia were obtained from the cultures of "Champignon x" of Foex, and shown to be identical with the American fungus (Sprague & Fellows, 1934). Subsequently the pathogen has been identified in most countries where wheat is cultivated (Davies, 1970). It was first reported in the U.K. in 1936 (Glynne, 1936).

Deighton (1973), reviewing Cercospora and related genera, reclassified the fungus on the basis of spore characteristics, as Pseudocercosporella herpotrichoides, placing it in a new genus. In the most recent taxonomic review of the species, Nirenberg (1981) recognised two major varieties, P. h. var. herpotrichoides

and P. h. var. acuformis, on the basis of morphological differences, and identified two new species P. anguioides and P. aestiva. All were pathogenic on cereals. The two varieties of P. herpotrichoides, var. herpotrichoides and var. acuformis, roughly corresponded to the two main pathogenic types, wheat (W) and rye (R), repectively, which had been recognised previously (Brown & Griffin, 1983).

P. herpotrichoides is an imperfect fungus, for which no sexual state has been found. Furthermore, although a diploid strain has been produced in vitro and mitotic reassortment of the genetic material was presumed to occur (Davies & Jones, 1970a), a parasexual cycle has yet to be demonstrated.

2.3 PATHOLOGY

2.3.1 Host Range and Symptom Development

There is a considerable literature on the phytopathology of P. herpotrichoides, covering all aspects of its epidemiology and disease control. The host range of this pathogen is quite extensive; in addition to the major cereals wheat and barley, and to a lesser extent oats, P. herpotrichoides is also pathogenic on rye and many other members of the Gramineae (Booth & Waller, 1973; Cunningham, 1965; Hartz, 1969; McKay et al, 1956; Scott et al, 1975, 1976; Sprague & Fellows, 1934).

Infection typically occurs at the stem base, usually below the first node, and an elongated lesion develops. The fungus successively penetrates the leaf sheaths ultimately filling the

stem lumen with mycelium (Sprague & Fellows, 1934; Lange-de la Camp, 1966a). The lesion may girdle the stem, killing the phloem and inducing premature maturation and "whiteheads". Alternatively the stem is weakened leading to random lodging or "straggling" (Sprague & Fellows, 1934). The fungus also causes a post-emergence foot rot which may kill seedling plants or tillers.

Reduction in yield results both from the direct effects of the pathogen on the host plant and indirectly by increasing lodging and storm damage (Ponchet, 1959). Infection in wheat and barley reduced both grain number per ear and thousand grain weight and infected plants produced fewer tillers (Ponchet, 1959; Davies & Jones, 1970b; Jordan et al, 1979; Clarkson, 1981).

2.3.2 Epidemiology

The disease corresponds to the description of a simple interest disease (Rowe & Powelson, 1973b). Infection occurs during cool, wet periods in autumn and early spring, from spores produced on contaminated stubble debris from a previous crop (Sprague & Fellows, 1934; Ponchet, 1959; Diercks, 1966). Young lesions do not usually sporulate and so do not act as a source of inoculum for secondary infections, and spore production from stubble trash usually declines after the end of April, coinciding with a reduction in the incidence of new infections (Diercks, 1966; Hollins & Scott, 1980). Consequently the amount of disease present in a crop depends on the climatic conditions prevailing early in the season. Sporulation on infected straw, under controlled conditions, occurred between 0 and 20°C with the most consistent production within the range 1 - 15°C (Moritz &

Brockman, 1933; Glynne, 1953; Jorgensen, 1964; Rowe & Powelson, 1973a). Glynne (1953) found that fluctuating temperatures between -3 and 13 °C increased spore formation from straw; while in pure culture moderate temperatures (9 - 17°C) and exposure to near ultraviolet light enhanced sporulation by the fungus (Leach, 1967; Ward & Friend, 1979; Reinecke & Fokkema, 1979). In all cases high humidity was essential, although excess moisture inhibited the sporing process (Glynne, 1953).

The environmental conditions required for infection have been thoroughly investigated (Dickens, 1958; Defosse, 1966, 1967). Lange-de la Camp (1966b, 1967) found that the temperature optimum for infection depended less on the particular isolate used, than on the cereal species and variety, infection of wheat plants decreased between 8°C and 15°C. Similarly Schrodter and Fehrmann (1971) identified an optimum of between 8 and 9°C for wheat infection, and found that a period of at least 15 h with over 80% air humidity was also essential. In wheat seedlings the rate of penetration of successive leaf sheaths was found to increase over the range 6 to 18°C, coinciding with the increase in radial growth rate of the fungus, *in vitro* (Scott, 1971). The low temperature requirement for spore production and infection undoubtedly explains why most field infection occurs during the autumn and spring periods.

Lesions develop throughout the summer, particularly in cool wet weather, and infected stubble remains in the field after harvest. Dense mats of polygonal cells, or stromata, are formed in the infected tissues (Ponchet, 1959), which serve as resting

structures, enabling the pathogen to survive for up to two years in the absence of a crop (Cox & Cock, 1962). The cellulolytic ability of P. herpotrichoides is limited, and as a result the rate of utilization of the nutrient resources of the straw is low (Hanssler, 1972; Garrett, 1975). Consequently the mycelium can remain viable for relatively long periods during this saprophytic phase, retaining the ability to sporulate and thereby to initiate a new disease cycle (Blair, 1954; Macer, 1961a, b; Deacon, 1973a). Spore dispersal occurs by rain splash and so is restricted to quite short distances (Fitt & Nijman, 1983). There is, therefore, little movement of inoculum between fields (Fehrmann & Schrodter, 1971; Rowe & Powelson, 1973b).

2.3.3 Control

Before the introduction of the systemic benzimidazole fungicides control of eyespot involved cultural rather than chemical measures. Many chemicals were tested for efficacy against P. herpotrichoides, and although some did cause a reduction in disease severity, none were commercially successful (Sprague & Fellows, 1934; Davies, 1970).

Crop rotation, with at least two seasons between successive cereal crops, was recommended, since the fungus is able to survive in debris from previous crops for up to two years. Furthermore, because P. herpotrichoides competes poorly with other soil micro-organisms (Macer, 1961a; Deacon, 1973b) and so is unable to infect plants by growing through the soil, farmers were encouraged to plough-in infected stubble, to prevent spore formation on exposed straw (Fehrmann & Schrodter, 1971). Stubble

burning was found not to reduce the incidence of the disease presumably because most lesions occur at ground level and so escape destruction (Sprague & Fellows, 1934; Slope et al, 1970). Sowing date also affects the rate of disease build-up, the earlier the time of sowing the more severe the level of infection the following season (Sprague & Fellows, 1934; Davies, 1970).

Use of the growth-regulatory chemical Cycocel was found to reduce the loss of yield caused by the disease. Investigation has shown that this was mainly due to the effect of this compound on the plant, causing stem shortening and thickening of the haulm base. In consequence both the rate of penetration of the stem by the fungus, and the degree of lodging caused by the disease, was reduced (Slope et al, 1969).

Host resistance to attack by P. herpotrichoides has been identified in wild cereal species and in some cultivars. In wheat, inherent resistance was first identified in cv. Cappelle Desprez and has been used in breeding programmes to produce other resistant wheats (Bruehl, 1983). Resistance is polygenic, and appears to involve changes in the degree and timing of secondary cell wall thickening in the stem, as well as a hypersensitive reaction in the resistant plants (Law et al, 1976; Murray & Bruehl, 1983; Guillot-Salomon et al, 1981; Bateman & Taylor, 1976; Kahn & Bouriquet, 1984; Souilie et al, 1985).

The effectiveness of the benzimidazole fungicides for the control of P. herpotrichoides was soon recognised (Catling, 1970). Benomyl, carbendazim, thiabendazole and thiophanate-methyl have all been used commercially against this pathogen (Taylor &

Waterhouse, 1975; Rule, 1975; Bruehl et al, 1982). The usual method of application of these fungicides is by foliar sprays, although seed treatment has been shown to give reasonable control (Lemaire et al, 1970; Davies, 1970). A single spray per season has usually been found to be sufficient for control, applied either in the late Autumn or in Spring, depending on the rate of infection in the immature crop (Jordan & Tarr, 1981).

With the emergence of resistance to this group of fungicides in the eyespot pathogen (Rashid & Schlosser, 1977), alternative antifungal agents were sought, amongst which the ergosterol biosynthesis inhibitor prochloraz has been found to be very effective. This compound is now marketed for use against eyespot in combination with carbendazim in an attempt to extend the useful life of these fungicides (Giffiths et al, 1983).

2.4 MORPHOLOGY AND VARIATION IN P. HERPOTRICHOIDES

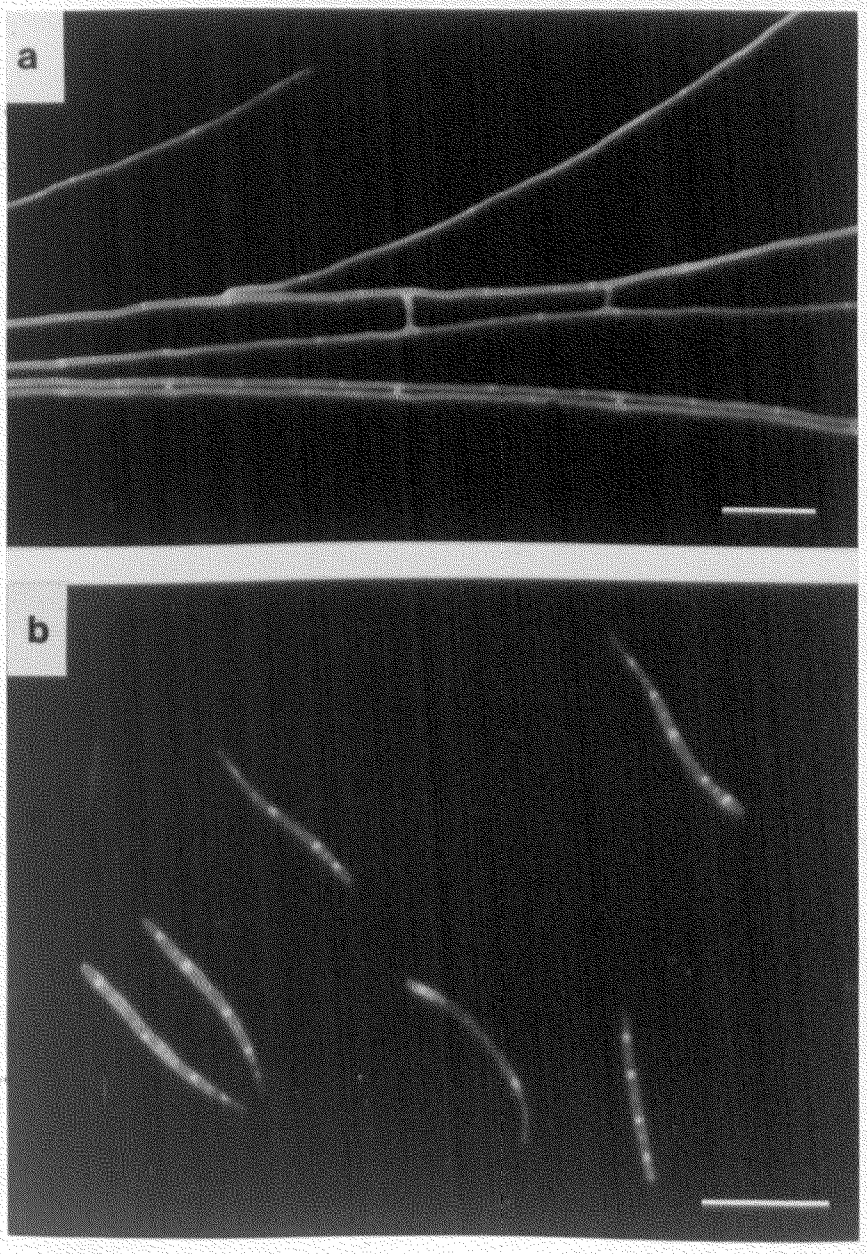
2.4.1 Cytology

The hyphal diameter in P. herpotrichoides is relatively small, hyphae often growing in close association, in parallel lines with many interhyphal connections (Fig 2.1a). Anastomosis between hyphae belonging to the same mycelium occurs frequently and nuclear migration through the interhyphal bridges has been observed (Lange-de la Camp, 1964). The hyphae are ensheathed in a layer of mucilage (Reiss, 1971), which presumably aids their adherence to the substrate and leads to coherence of the mycelium in liquid culture (Hanssler, 1972).

The mycelium of P. herpotrichoides is septate, each cell containing a single nucleus (Lange-de la Camp, 1964; Deacon,

Figure 2.1 a Regular hyphal anastomosis in P.herpotrichoides (Isolate 22-8) form bridges between neighbouring hyphae. Mycelium fixed with 70% ethanol was stained with tinopal (0.01% aqueous solution) and viewed under UV-fluorescence. Bar represents 20 µm.

Figure 2.1 b Conidia of P. herpotrichoides are typica. multicellular, each cell containing a single nucleus. Spores fixed with 70% ethanol, nuclei stained with chromomycin A_3 and septa counterstained with tinopal. Bar represents 20 µm.


a
b

1973b). Apical cells, however, usually contain two or more nuclei, so a heterokaryotic mycelium could theoretically be maintained. The conidia are hyaline and multicellular, typically with 5-7 uninucleate cells (Fig 2.1b). The spores are formed from individual, intercalary mother cells, in consequence the nuclei are all derived from a single mother cell nucleus, and so, except for spontaneous mutational events during conidiogenesis, are genetically identical (Glynne, 1953; Davies, 1970). Conidia may also be produced by pseudoparenchymatous stromata cells, these are similarly uninucleate and so the conidia will also contain identical nuclei (Deacon, 1973b).

2.4.2 Cultural Morphology

Variation in colony morphology in P. herpotrichoides, in pure culture, is well known. Various attempts have been made to categorise the different morphological types and associate them with other characteristics, particularly with pathogenicity (Lange-de la Camp, 1966b; Scott et al, 1975). Lange-de la Camp (1966a) recognised two major colonial forms in culture, one with a faster growth rate and even margin, the other with slower growth and an irregular colony edge (Fig 2.2). In pathogenicity studies of these strains, it was found that they corresponded to two virulence types; the fast growing, even-edged isolates were much less pathogenic to rye than wheat, while the slower, irregularly-edged isolates were equally pathogenic to rye and wheat. Consequently they were designated wheat types (W- or BW-types) and rye types (R- or BWR-types), respectively (Lange-de la Camp, 1966b; Scott et al, 1975).

The two varieties, P. h. var. herpotrichoides and P. h. var. acuformis, described by Nirenberg (1981) display cultural morphologies which roughly correspond with the two pathogenicity types reported by previous authors. Whilst no comparison of the virulence of the varieties on different cereal hosts has been made, physiological differences have been demonstrated (Nirenberg, 1984). For example, hyphal anastomoses occurred between isolates belonging to the same variety but were not seen between isolates of different varieties or between these varieties and either of the two new species, P. aestiva and P. anguioides, identified by Nirenberg (Fig. 2.2).

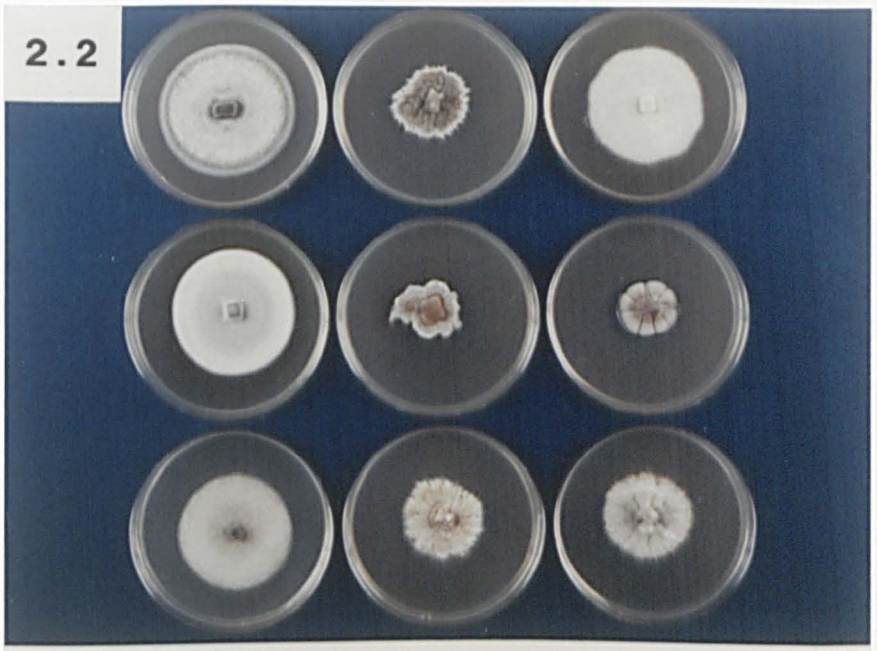
Variation in sensitivity to carbendazim was also found between these taxonomic groups; isolates of P. h. var. herpotrichoides were generally slightly more sensitive to this benzimidazole fungigide than those of P. h. var. acuformis. The basis for these differences was not identified (Nirenberg, 1984). It is unlikely, however, that the observed variation in basal sensitivity alters the potential for aquired, mutational resistance to carbendazim, since Brown et al (1984) isolated both wheat and rye types of the eyespot pathogen with resistance to these compounds.

2.4.3 Growth Rate Studies

The radial growth of four isolates, two BW-types and two BWR-types, on PDA is shown in Fig. 2.3, and demonstrates the difference between the morphological types. The results of a more extensive assessment of radial growth rate is presented in Table 2.1. Significant differences were found in growth rate both

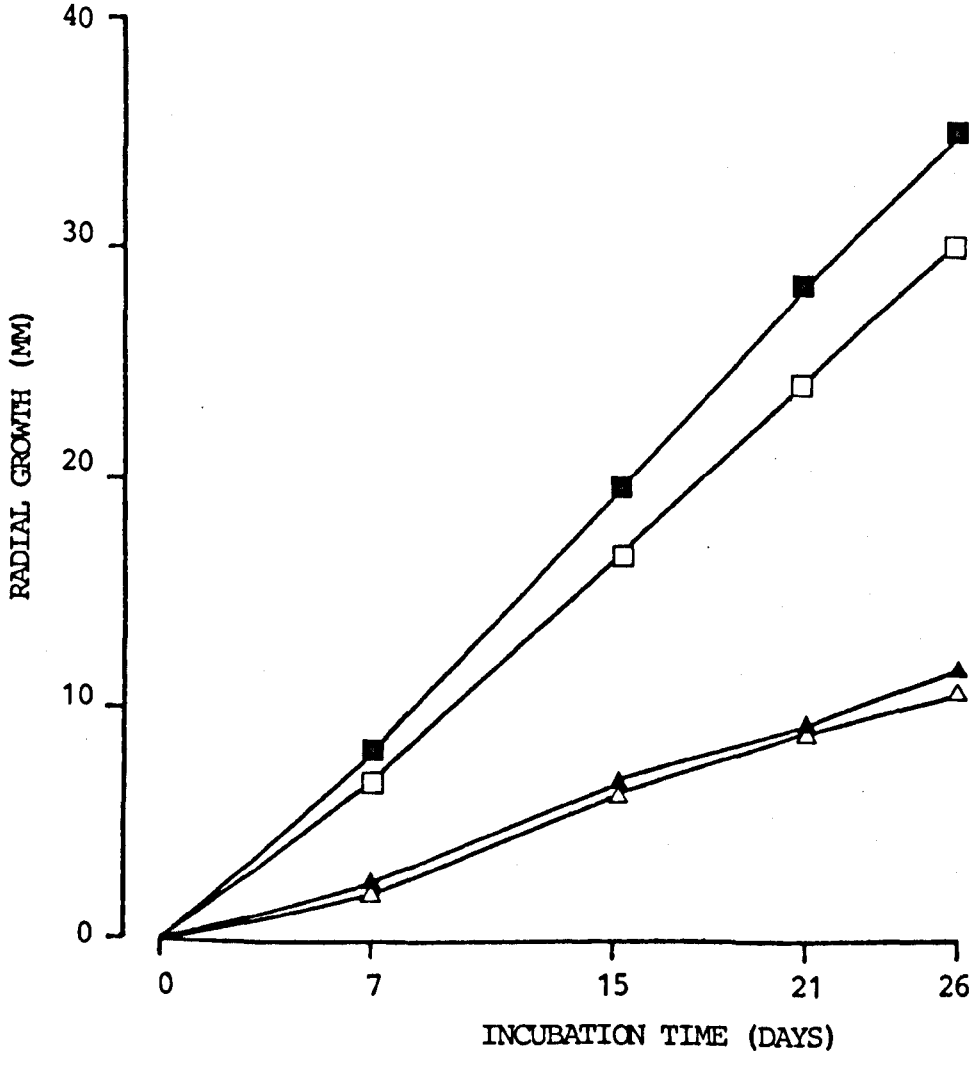
Figure 2.2 Variation in colony morphology in Pseudocercosporella. Left to right: top row - 22-20, P. herpotrichoides BW-type; 22-119, P. herpotrichoides BWR-type; 24-1, P. anguoides. Middle row: 22-5, P. herpotrichoides BW-type; 22-17, P. herpotrichoides BWR-type; 23-1, P. aestiva. Bottom row: 22-2, P. herpotrichoides BW-type; 22-4, P. herpotrichoides BWR-type; 22-116, P.h. var. acuformis type-isolate. Colonies growing on MYG for 25 days at 19°C.

Figure 2.4 Morphological instability in the BWR-type isolate 22-9. Colony grown on MYG for 25 days at 19°C.


2.2


2.4

Figure 2.3 Radial growth (mm) of two BW-type isolates, 22-1 (■) and 22-2 (□), and two BWR-type isolates, 22-3 (▲) and 22-4 (△) at 19°C on PDA (15 ml/9 cm diameter Petri-dish) measured over 26 days.



within and between the two morphological types of *P. herpotrichoides*. Excluding isolate 22-3, all BWR-types had a significantly slower rate of growth ($P = 0.01$) than the BW-type strains. Isolate 22-116, the *P. h.* var *acuformis* isolate, had a rate of growth equivalent to the fastest of the BWR-type isolates. The growth rate of the isolate of *P. anguoides*, 24-1, was intermediate between the BW- and BWR-types of *P. herpotrichoides*; while *P. aestiva* (23-1) was relatively slow growing, comparable with the slowest BWR-types. BWR-type isolate 22-3 showed a considerable increase in growth rate in the interval between the two assessments (cf. Fig. 2.3 and Table 2.1), even though cultures were recovered from straw stock culture for each test. This apparent instability will be discussed later.

2.4.4 Spore production

Great variation in the capacity of isolates to sporulate under controlled conditions was observed, some isolates failing to produce spores on TWA, even after prolonged incubated at 19°C in the dark. The stimulatory effect of near-UV light ("Black light") on spore formation in *P. herpotrichoides* has been reported (Leach, 1967). In an attempt to improve conidial production, sporulation under near-UV light and dark conditions was compared. The effects of near-UV light on sporulation was investigated by incubating cultures, growing on TWA in plastic Petri dishes in a light-proof box, fitted with two near-UV light tubes, 30 cm above the plates. Spore numbers were determined after 18 days under continuous near-UV light at 16 to 18°C, or

Table 2.1 Comparison of growth rates of *Pseudocercosporella* isolates. Colonies grown on MYG agar (20 ml 9 cm diameter plate-1) at 19°C, radial growth rates (mm day^{-1}) determined after 8 days.

	ISOLATE NUMBER	RADIAL GROWTH RATE (mm day^{-1})	P = 0.05	P = 0.01
w	22-21	1.40	a*	a*
w	22-1	1.22	b	b
w	22-5	0.98	c	c
w	22-118	0.97	c	c
w	22-20	0.94	c	c
w	22-22	0.88	d	d
w	22-18	0.87	d	d
w	22-2	0.86	d	d
x	22-3	0.79	e	de
y	24-1	0.79	e	de
z	22-8/1	0.79	e	de
y	22-116	0.74	e	ef
x	22-121	0.67	f	fg
x	22-183	0.66	f	fg
x	22-10	0.63	fg	gh
x	22-8	0.58	gh	ghi
x	22-12	0.55	hi	hij
x	22-7	0.54	hi	hij
x	22-16	0.50	ij	ijk
x	22-119	0.49	ij	ijk
x	22-120	0.48	ij	ijk
x	22-117	0.48	ij	ijk
x	22-4	0.48	ij	jk
y	23-1	0.46	ij	jk
x	22-17	0.42	j	k

w *P.herpotrichoides* BW-type isolates.
x *P. herpotrichoides* BWR-type isolates.
y *P. h.* var. *acuformis* (22-116); *P. anguoides* (24-1); *P. aestiva* (23-1).
z Sector of 22-8, with a fast, even-edged growth morphology.
* Isolates with the same letter not significantly different for growth rate at either the 5% (P = 0.05) or 1% (P = 0.01) levelcalculated using multiple range test (Duncan, 1955)

dark at 19 °C. The effect of transferring plates from near-UV to dark conditions or vice versa after 9 days was also assessed (Table 2.2).

The five isolates studied varied greatly in their response to the different light and temperature regimes. Only one (22-16) showed significant stimulation of spore production by near-UV light. Under continuous near-UV light sporulation of all isolates except 22-20 was reduced. However, the use of alternating dark and near-UV, possibly for shorter intervals may yet prove useful to induce spore formation in recalcitrant strains.

Differential spore production may be important when preparing spore inocula from mixed cultures, as is often the case when screening for new antifungal compounds in plant tests.

2.4.5 Instability

Throughout the course of this work it was observed that BWR-type isolates had a tendency to produce fast-growing, even-edged sectors, more characteristic of BW-type growth, from colonies on agar medium. These sectors were frequently stable, retaining the new morphology when transferred successively to fresh plates (Fig 2.4). Since differences in cultural morphology have been widely used as an important characteristic distinguishing between the two pathogenic types of P. herpotrichoides, in studies of changes in field populations of the pathogen, this feature is disturbing. Consequently a number of these variants were studied further.

Table 2.2 Spore production under different regimes of dark and near-UV light. Spore numbers (mm^{-2} agar surface) are the mean of three estimations for each treatment/isolate combination.

ISOLATE NUMBER	LIGHT REGIME*	SPORE NUMBERS ($x10^3$ mm^{-2})
22-6	1	7.0
	2	4.1
	3	0.1
	4	2.6
22-8	1	9.5
	2	2.2
	3	0.0
	4	2.4
22-16	1	0.9
	2	0.4
	3	0.0
	4	4.9
22-20	1	14.6
	2	18.9
	3	16.0
	4	16.3
22-116	1	6.9
	2	4.1
	3	0.0
	4	2.7

* 1 Maintained at 19°C in the dark
2 Exposed to near-UV light for 9 days at 16-18°C, then tranferred to the dark at 19°C for 9 days.
3 Maintained under continuous near-UV light at 16-18°C for 18 days.
4 Incubated at 19°C in the dark for 9 days, then transferred under near-UV light at 16-18°C for 9 days.

Table 2.2 continued

Analysis of Variance

SOURCE	DF	SS	MS	F	
Isolates	4	196.557	49,139	17.44	P = 0.001
Light regimes within isolates	15	42.275	2.818	2.36	P = 0.025
Replicates (residual)	40	47.724	1.193		
Totals	59	286.557			

2.4.5.1 Isolate 22-8

One such faster-growing sector was obtained from the BWR-type isolate 22-8, and was designated 22-8/1. This variant differed in several respects from its progenitor isolate, including growth rate (Table 2.1), abundance of aerial mycelium and pigmentation (Fig. 2.5). The growth rate of 22-8/1, while significantly greater than 22-8 (P = 0.01), was equivalent to that of isolate 22-3 and the P. h. var. acuformis type-isolate 22-116.

The obvious explanation for the appearance of this atypical form, that it is the result of contamination by one of the BW-type isolates being used, can be readily discounted. The benzimidazole sensitivity of the "parental" isolate, 22-8, was quite characteristic, being highly resistant to carbendazim and only moderately resistant to thiabendazole (See Chapter 3.0). This phenotype was retained in 22-8/1, and did not occur in any of the benzimidazole-resistant BW-type isolates in the collection.

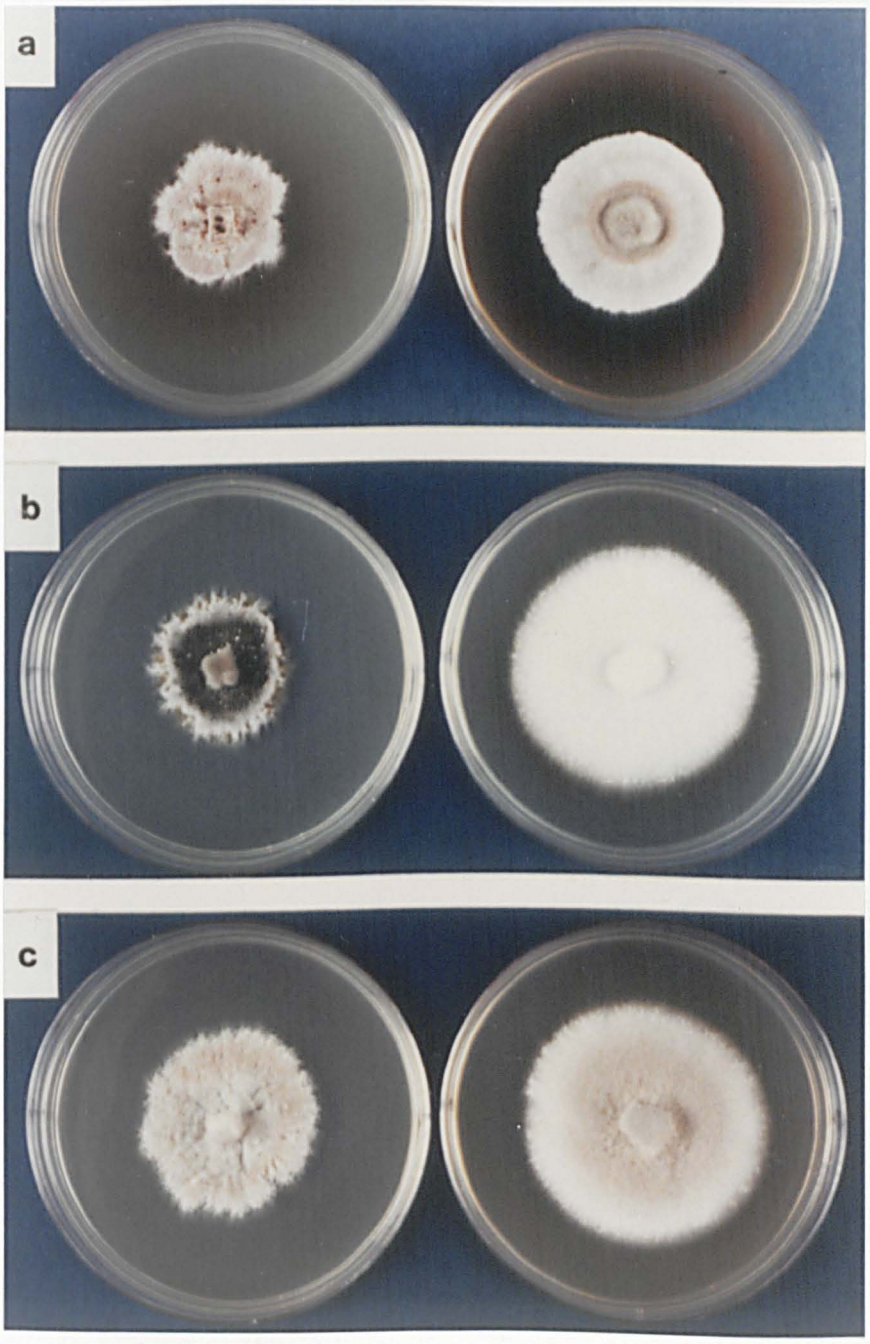
Figure 2.5 Morphological instability in BWR-type P. herpotrichoides isolates. Original colony types (left) and fast-growing, even-edged sectors (right) derived from them.

a 22-8 & 22-8/1

b 22-9 & 22-9 sector

c 22-15 & 22-15 sector

Colonies grown on MYG for 27 days at 19°C.


a
b
c

While its sensitivity to benzimidazole fungicides was unchanged compared with 22-8, there was evidence that it was altered in its response to the ergosterol biosynthesis inhibitor, fenpropimorph (Table 2.3). However, faster growing variants of two other isolates, 22-9 and 22-15, did not show the same difference. Sensitivity to prochloraz, another EBI fungicide was unaltered.

TABLE 2.3 Fungicide Sensitivity of isolates 22-8 and 22-8/1, and of variant types of two other BWR-type isolates. ED values (μM) for growth inhibition determined after 8 days at 19°C.

	FUNGICIDE RESPONSE					
	FENPROPIMORPH			PROCHLORAZ		
ISOLATE*	ED_{50}	ED_{75}	ED_{90}	ED_{50}	ED_{75}	ED_{90}
22-8	<10	39	140	<0.2	<0.2	<0.2
22-8/1	39	162	197	<0.2	<0.2	<0.2
22-15/1	<10	39	63	<0.2	<0.2	<0.2
22-15/2	<10	32	52	<0.2	<0.2	<0.2
22-9/1	11	100	155	<0.2	<0.2	0.2
22-9/2	20	66	143	<0.2	<0.2	0.2

* 22-15/1 and 22-15/2 were single spore derived colonies of the BWR-type isolate 22-15. The growth rate of 22-15/1 on fungicide free MYG agar was 0.67 mm day^{-1} and that of 22-15/2 was significantly faster ($P = 0.01$) at 0.70 mm day^{-1}

22-9/1 and 22-9/2 were single spore derived colonies from 22-9. The growth rates of which, on fungicide free medium, were 0.70 and 0.60mm day^{-1} respectively ($P = 0.01$).

2.4.5.2 Isolate 22-15

Another BW-type isolate in which fast-growing sectors were isolated was 22-15 (Fig 2.5). Colonies arising from single spores of this isolate, which had itself originally been purified by single spore isolation, also exhibited considerable variation in appearance particularly on MYG agar. Some were dark brown in pigmentation with a more or less regular margin, while others were much lighter coloured with either an even or feathery edge (Fig. 2.6). These differing forms were initially obtained on MYG containing thiabendazole, however, the presence of this inhibitor did not appear to have induced the variation and did not affect the frequency with which they were recovered since they could be isolated on fungicide-free medium in the same relative proportions (Table 2.4). On TWA differences in sporing ability, abundance of aerial mycelium, pigmentation and stroma development were apparent (Table 2.5), both between the white pigmented and brown pigmented types and within each of these groupings. Generally spore production was not observed in colonies showing abundant aerial mycelium, hyphal pigmentation or stroma development, while colonies derived from brown colonies sporulated more readily than those from white colonies. On MYG agar some indication of further instability was apparent, many of the brown pigmented colonies producing white sectors which, when transferred onto fresh medium, were identical to the white pigmented colonies previously obtained. Darker areas were seen within some of the white colonies but could not be isolated. Single spore isolates taken from several brown and white colonies

generally retained the phenotype of the original colony (Table 2.6). Spores from one brown colony, however, produced brown and white colonies in equal proportion.

TABLE 2.4 Frequency of isolation of morphological variants as single spore isolates from isolate 22-15.

RECOVERY MEDIUM	COLONY PHENOTYPE			TOTAL NUMBER OF COLONIES SCREENED
	WHITE*	BROWN**	UNCLASSIFIED	
MYG AGAR 0μM TBZ	32 (70)***	12 (26)	2 (4)	46
MYG AGAR 1μM TBZ	106 (75)	32 (23)	3 (2)	141
MYG AGAR 5μM TBZ	109 (78)	24 (17)	7 (5)	140
TOTALS	247 (75)	68 (21)	12 (4)	327

* White pigmented colonies with even or feathery margin.
** Brown pigmented colonies with even margin
*** Figures in parentheses indicate proportion of isolates in each catagory as a percentage of the total number screened

Table 2.5 Characteristics of 22-15 variant types on TWA. Colonies assessed visually after 15 days growth at 19°C

	CHARACTER ASSESSMENT*				
COLONY TYPE ON MYG	SPORULATION	MYCELIAL PIGMENTATION	AERIAL MYCELIUM	STROMA DEVELOPMENT	NUMBER IN EACH GROUP
White,	-	-	-	-	4
even or	-	-	++	-/+	6
feathery-	-	-	+++	-/+	6
edged	-	+	++	-/+	7
	-	+	++/+++	++/+++	4
	+/++	-	-	-	14
Total					41
Brown,	-	+++	-	-	5
even-	+	+	-	-	1
edged	++/+++	-	-	-	14
Total					20
Total number of colonies screened					61

* Characters scored visually as follows:
\- no sporulation/pigmentation/aerial mycelium/stroma development apparent.
\+ slight " " " " apparent
++ moderate " " " " apparent
+++ considerable " " " " apparent

Table 2.6 Stability of variant phenotypes from 22-15. Appearance of colonies derived from single spores of white- or brown-pigmented variants.

PARENTAL PHENOTYPE	COLONY NUMBER	NUMBER OF RE-ISOLATES OF EACH TYPE		TOTAL NUMBER SCREENED
		WHITE	BROWN	
Brown, even-edged	1	0	104	104
	2	0	104	104
	3	0	104	104
	4	0	104	104
	5	0	104	104
	6	0	104	104
	7	49	55	104
	8	0	71	71
White, even- or feathery-edged	9	104	0	104
	10	78	0	78
	11	104	0	104
	12	104	0	104
	13	103	0	103
	14	104	0	104
	15	104	0	104

2.5 ANTAGONISM

Pseudocercosporella herpotrichoides is widely reported to be a poor competitor in the soil environment (Macer, 1961a, b; Garrett, 1975). The long term survival of the fungus in the soil has been related to the formation of resting stuctures (stromata) in contaminated straw, and the low cellulolytic rate of the pathogen (Macer, 1961b). In addition to these properties, the survival potential of this pathogen is increased by its ability to actively inhibit or antagonise other cereal foot rot fungi, particularly Rhizoctonia cerealis, the sharp eyespot pathogen (Kapoor & Hoffmann, 1984). Consequently applications of

benzimidazole fungicides to reduce the incidence of P. herpotrichoides can result in an increase in the frequency of the sharp eyespot fungus (Reinecke et al, 1979).

In culture some isolates of P. herpotrichoides show inhibition for growth of other strains of the fungus, resulting in a definite zone of inhibition on the side of the colony nearest the antagonistic isolate. This phenomenon is not exhibited by all strains and does not appear to affect all other isolates equally. The biochemical basis of the inhibition remains to be studied.

2.6 DISCUSSION

The relationship between the two morphological types of P. herpotrichoides (BW and BWR) is not clear, both occur in the same fields, and can be isolated from the same lesion (M. Griffin, pers comm.) Bateman et al (1985) failed to find mixed infections in a survey of wheat crops, possibly as a result of the isolation technique employed, which involved serial transfers of mycelium obtained from infected stems on agar medium and the isolation of hyphal tips. These manipulations would preferentially select for the fastest growing mycelium, consequently mostly BW-type isolates were recovered.

The system most extensively used to differentiate the two pathotypes is based on the morphology of colonies in agar culture. The variation in growth rate and the phenomenon of instability, which was seen in almost all the BWR-types studied,

including 22-116 the P. h. var acuformis isolate, may considerably confuse this distinction. Obviously a greatly increased number of characters for the differentiation of these types is required, possibly involving biochemical characterisation. Furthermore the true status of the pathotypes in relation to each other, and to the described taxonomic varieties urgently needs further clarification.

Strain instability, usually recognised as variation in cultural morphology but also affecting physiological characteristics and pathogenicity, is a common feature of many fungi (Hansen, 1938; Ou & Ayad, 1968; Grindle, 1979; Hastie & Heale, 1984). The basis of the strain instability observed in P. herpotrichoides remains unclear. Various mechanisms for the origin of the variation, however, may be suggested including heterokaryosis, mutation and ploidy changes.

Heterokaryosis has long been recognised as an important potential source of variation in fungi (Davis, 1966). Changes in the ratio of the component nuclei in heterokaryons have been related to the "dual phenomenon" in Botrytis cinerea and other fungi, where repeated single spore isolation yields types with differing morphological characteristics as a result of selection for the component homokaryons (Hansen, 1938). Similarly, variation in the level of botran resistance in conidial progeny of resistant strains of B. cinerea has been attributed to changes in the proportion of nuclei conferring resistance or sensitivity to the fungicide in heterokaryotic mycelium (Webster et al, 1970).

Segregation of cytoplasmic factors can also result in

cultural variability. Jinks (1959) showed that in four wild-type isolates of Penicillium exhibiting morphological instability two were heterokaryons while the other two were heteroplasmons. Grindle (1979) suggested that while some of the variation found in conidial and hyphal derivatives of isolates of B. cinerea could be due to the segregation of nuclei in heterokaryons, selection for cytoplasmic genetic elements may also be involved. Furthermore, the loss of, or mutation in, a cytoplasmically inherited factor has been shown to be the basis of the spontaneous hyaline, non-sclerotial sectors readily obtained in dark-pigmented, sclerotial isolates of Verticillium albo-atrum and V. dahliae (Typas & Heale, 1976; Hastie, 1981).

In Nectria haematococca two forms of morphological variation have been described: atypical hyphal growth occurring at the colony edge forms either V-shaped sectors as the colony grows or spreads around the perimeter to form a continuous ring. Interactions between six nuclear genes, two cytoplasmic factors of unknown molecular nature and environmental conditions have been implicated in the expression of this instability (Parisot et al, 1981).

Apparent instability in culture may also result from mutation. Numerous mutations leading to modified colonial morphology have been induced in Neurospora crassa and Aspergillus nidulans (Fincham et al, 1979). Many of these affect hyphal branching pattern, and a few are associated with deficiencies for particular enzymes in carbon metabolism. Mutation may also lead to differences in pigmentation; most mutagenic programmes with

fungi readily generating colour mutants (Fincham et al, 1979). Such mutations may affect both the production of particular pigments or their intra- and extra-cellular distribution (Parisot et al, 1981, 1984; Hastie & Heale, 1984; Avalos et al, 1985). Nasim & Auerbach (1967) investigated mutational instability in Schizosaccharomyces pombe in which a proportion of cells treated with one of a range of mutagens gave rise to mosaic colonies repeatedly on sub-culture. Mosaic colonies ariseas a result of a mutational event affecting only one strand of the DNA duplex. Consequently DNA-replication and nuclear division produces daughter cells half of which are wild-type and half mutant. Sub-culture of these daughter cells usually yields pure mutant or wild-type colonies, occasionally, however, mosaic colonies are generated persistently. The basis of this phenomenon appears to be instability of replication at specific sites in the DNA, though the mechanism is not fully understood (Fincham et al, 1979).

Heteroploidy, the generation of aneuploid and euploid series from haploid or diploid nuclei, has been suggested as a significant source of variability in fungi (Tolmsoff, 1983). Ploidy changes, associated with cell differentiation or uneven nuclear division, may yield a great range of aneuploid and euploid types each with its own particular phenotype. The variation in colony morphology, pigmentation and pathogenicity observed in haploid microsclerotial derivatives in V. albo-atrum was assumed to result from chromosome reassortment and gene repression and derepression occurring in homozygous diploid microsclerotial cells (Tolmsoff, 1972). As the chromosomes of

most fungi are too small and diffuse for accurate determination of their number by cytological means, much of the evidence for heteroploidy in fungi relies on the variation in the DNA-content of nuclei. Not only are large differences observed between different wild strains but within individual isolates estimates of DNA-content may vary considerably, particularly between different cell types (Tolmsoff, 1983). In Pyricularia oryzae, a particularly variable species, both heteroploidy and parasexual recombination have been implicated in strain instability (Ou, 1980; Tolmsoff, 1983; Genovesi & MaGill, 1976), although these two mechanisms are obviously not mutually exclusive.

Heterokaryosis is unlikely to be the cause of strain instability in P. herpotrichoides since the fungus is composed of predominantly uninucleate mycelium, and the original isolates were obtained as single spore-derived colonies in order to exclude the possibility of using mixed cultures. Furthermore, the benzimidazole response of variant sectors was identical to that of the progenitor colonies, and sectors from auxotrophically marked BWR-type strains retained the nutritional requirement of the "parent". The extent to which the other mechanisms described are involved remains to be investigated.

CHAPTER 3

RESISTANCE TO FUNGICIDES IN *PSEUDOCERCOSPORELLA HERPOTRICHOIDES*

3.1 Introduction

To understand the potential risk of failure of disease control due to resistance development, and in order to develop new strategies for control information is required in several key areas. The ability of the pathogen to develop resistance, the nature of the resistant isolates and the potential variation in resistance expression must be determined. Knowledge of the level of resistance and the expression of cross-resistance to other fungicides is also of vital importance. In addition, pleiotropic effects of resistance, particularly on fitness (viability, spore production and growth rate) and pathogenicity will allow some estimate of the likely persistence of resistant strains in the absence of the fungicide and hence the possibility of reducing the frequency of resistant isolates simply by reducing the use of the fungicide.

The use of *in vitro* assays, usually involving measurement of growth, is the most common method by which fungicide sensitivity is determined. However, this technique does have a number of limitations, which must be considered when attempting to relate the findings of such studies to the practical application of disease control in the field. For example, the nature and availability of the toxicant may well be different in the plant than in agar or liquid media, and the response of the pathogen correspondingly different. Furthermore, *in vitro* assays rarely allow any greater assessment of the ability of the

pathogen isolate to cause disease than the arbitrary estimate of fitness obtained from measurements of growth rate and spore production under the conditions of the test.

Such tests do, however, permit the investigation of resistance expression in the pathogen under controlled conditions, information which may prove invaluable in the development of new disease control strategies in the field. Similarly the use of laboratory mutants to investigate aspects of resistance expression, enables not only an assessment of the potential for variation in the fungus, but also a comparison of fungicide sensitivities against a more homogeneous genetic background than is obtained by studying a necessarily diverse collection of field isolates.

Resistance to the benzimidazole fungicides in P. herpotrichoides has led, since 1981, to loss of disease control with these chemicals in the UK (Brown et al, 1984). While many reports of resistance have been published little was known of the variation in the level of resistance or in the expression of cross-resistance to other mitotic inhibitors. Considerable interest has been shown in the phenomenon of negatively-correlated cross-resistance between benzimidazole fungicides and N-phenylcarbamate compounds (Kato et al ,1984 Leroux et al, 1985b), but the sucessful use of these drugs to control benzimidazole-resistant forms of the pathogen will depend on the nature of the relationship between the two groups of compounds, and the extent to which the fungus is able to overcome either or both. In an attempt to answer some of these questions the benzimidazole and phenylcarbamate responses of a range of field

isolates and laboratory mutants were determined *in vitro*. The results of these investigations are presented in this chapter.

The extensive use of ergosterol biosynthesis inhibiting (EBI) fungicides on cereal crops to control a variety of foliar diseases, and the increasing trend towards the cultivation of winter barley have both been implicated in the recently observed shift in the proportions of the two *P. herpotrichoides* pathotypes in field populations (Griffin, 1985; Bateman *et al*, 1985). This suggestion was supported by data showing a correlation between the frequency of particular pathotypes and the previous history of EBI useage (Griffin, 1985), and by *in vitro* tests of EBI sensitivity for a range of BW and BWR isolates from France (Griffin, 1985; Leroux & Gredt, 1985a,b). It was further suggested that the unequal distribution of carbendazim resistance between the two pathotypes, and the frequent predominance of BWR-types insensitive to the benzimidazole fungicides may be due to reduced sensitivity of these forms to the EBI compounds. In order to clarify this position the sensitivities of a number of field and laboratory isolates to a range of commercial and experimental EBI fungicides was assessed.

3.2 MATERIALS AND METHODS

3.2.1 Strains

The origin and characteristics of the field and type isolates used are given in Appendix I. Laboratory-induced resistance mutants were derived from isolates 22-20 and 22-12.

3.2.2 Determination of Fungicide Sensitivities

The sensitivity of isolates to a range of commercial and developmental fungicides was tested *in vitro*. Typically 3.5 to 4.0 mm diameter agar plugs, cut from the edge of growing colonies were placed onto MYG agar containing appropriate concentrations of the compound. Colony diameters, taken at right angles, measured after 8 to 14 days growth at 19° C, were used to calculate radial growth rates and expressed as a percentage compared with a control grown in the absence of the toxicant. Dose response curves were used to calculate the concentration of fungicide which reduced the growth rate by 50, 75 or 90 percent (ED_{50}, ED_{75} and ED_{90}), and the minimum concentration which completely inhibited growth (MIC). All determinations of sensitivity were performed at least in duplicate.

Once the range of concentrations needed to determine the sensitivity of strains had been established a simpler method of assessment was adopted in which the degree of growth inhibition was scored visually after 7 to 10 days, as follows:

- \- No growth
- \+ Growth restricted to the inoculum plug
- 1 Growth severely inhibited but mycelium in contact with the agar surface
- 2 Growth reduced to approximately 50% of the control
- 3 Growth rate comparable with the control

3.2.3 Isolation of Fungicide-Resistant Mutants

Mutants altered in their response to the benzimidazole fungicides were obtained both by selection for spontaneous mutants and after UV mutagenesis. Spontaneous resistance-mutants were isolated either by plating large numbers (>$5x10^7$ per plate) of conidia on MYG supplemented with an inhibitory concentration of the fungicide or as sectors of colonies from agar plugs on fungicide-containing medium.

UV-induced mutants were produced by exposing conidia to far-UV light (Philips Germicidal Lamp, 2.4 J m^{-2} s^{-1}) for up to 50 s. Irradiated spores were embedded or spread on medium containing either carbendazim or thiabendazole at an inhibitory concentration. Colonies appearing after 16 to 20 days were purified by fragmenting small quantities of mycelium in sterile water and plating onto fungicide-free MYG. Single colonies were tested for resistance to a range of benzimidazole and phenylcarbamate fungicides.

3.2.4 Fungicidal Compounds

The benzimidazole compounds carbendazim (methyl benzimidazol-2-ylcarbamate), benomyl (methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate) and thiophanate-methyl (dimethyl-4,4-(o-phenylene) bis(3-thioallophanate)were provided by Du Pont (UK) Ltd., Stevenage, and thiabendazole (2-(thiazol-4-yl) benzimidazole) by MSD Agvet. The N-phenylcarbamate, MDPC (methyl N-(3,5-dichlorophenyl)carbamate) was provided by Dr. D.W. Hollomon of Rothamsted Experimental Station and S32165 (1,1-

dimethylethyl N-(3,4-ethoxyphenyl)carbamate) was the generous gift of Sumitomo Chemical Company of Japan via Dr I. Saturo. All were technical grade (95 to 99% pure). Carbendazim, thiabendazole, MDPC and S32165 were dissolved in ethanol, benomyl and thiophanate-methyl in acetone, prior to addition to agar medium.

The EBI prochloraz (N-propyl-N-[2-(2,4,6-trichlorophenoxy) ethyl]imidazole-1-carboxamide; technical c.97% ai.) was the kind gift of Schering Agrochemicals, Chesterford Park, Cambridge. Fenpropimorph ((±)-cis-4-[3-(4-tert-butylphenyl) -2-methylpropyl] -2,6-dimethylmorpholine; Mistral, 75% ai.) was from May & Baker Ltd. Dagenham. Propiconazole ((±)-1-[2-(2,4-dichlorophenyl) -4-propyl-1,3-dioxolan-2-ylmethyl] -1H-1,2,4-triazole; technical 88% ai.) was provided by Ciba-Geigy Agrochemicals, Cambridge and triadimenol (1-(4-chlorophenoxy) -3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl) butanol; Bayfidan, 25% ai.) came from Bayer UK Ltd., Bury st. Edmunds. The experimental Du Pont compound DPX H6573 (bis(4-fluorophenyl) methyl(1H-1,2,4-triazol-1-yl methyl)silane, 40% ai. EC) was obtained from Dr. W. Carlisle of Trent Polytechnic, Nottingham.

Prochloraz and propiconazole were dissolved in acetone. All stock solutions were prepared at 200 times the required concentration, so that the final concentration of solvent in the medium never exceeded 0.5% (v/v).

Chemical structures and μM : μg ml^{-1} concentration conversion ratios for these fungicides are given in Appendix II.

3.3 RESULTS

3.3.1 Benzimidazole Sensitivity of Field Isolates

The sensitivity of thirty isolates of P. herpotrichoides, from various sources, to the benzimidazole fungicides carbendazim, benomyl, thiophanate-methyl and thiabendazole was assessed. Isolates were classified as either sensitive or resistant on the basis of the MIC for the four compounds (Table 3.1). Sensitive isolates were completely inhibited by 0.6 to 2.5 µM carbendazim, while the MIC for resistant isolates was greater than 1000 µM, the highest concentration used. In each case, where tested, resistance to carbendazim was associated with cross-resistance to the other three compounds. While MIC values for thiophanate-methyl and benomyl were similar to those for carbendazim, differences in the dose-responses of resistant isolates to these compounds were apparent (Figure 3.1).

The response of benzimidazole-sensitive isolates to the fungicides was much less variable, as the ED_{50} (Table 3.2) and dose response data (Figure 3.2) show. Variation in MIC values was observed between isolates but the concentration range used makes these differences appear greater.

Table 3.1 Benzimidazole sensitivity of field isolates: minimum inhibitory concentrations for carbendazim (MBC), benomyl (BEN), thiophanate-methyl (T-M) and thiabendazole (TBZ).

ISOLATE TYPE	ISOLATE NUMBER	MIC (μM) MBC	BEN	T-M	TBZ
BW	22-1	2.5	5	5	5
	22-5	1.0	5	10	5
	22-18	2.5	5	5	5
	22-19	2.5	5	10	3.5
	22-20	2.5	5	10	3
	22-22	1.0	1	5	5
	22-21	1.0	5	5	5
	22-2	>1000	>1000	>1000	>1000
	22-118	>1000	ND	ND	>1000
BWR	22-3	1.0	1	5	5
	22-12	0.6	1	5	2.5
	22-117	0.6	ND	ND	1.3
	22-120	0.6	ND	ND	2.5
	22-121	0.6	ND	ND	1.3
	22-6	>1000	>1000	>1000	20
	22-119	>1000	ND	ND	25
	22-4	>1000	>1000	>1000	50
	22-8	>1000	>1000	>1000	50
	22-13	>1000	>1000	>1000	50
	22-14	>1000	>1000	>1000	50
	22-17	>1000	>1000	>1000	50
	22-9	>1000	>1000	>1000	100
	22-7	>1000	>1000	>1000	500
	22-10	>1000	>1000	>1000	>1000
	22-11	>1000	>1000	>1000	>1000
	22-15	>1000	>1000	>1000	>1000
	22-16	>1000	>1000	>1000	>1000
	22-182	>1000	>1000	>1000	>1000
	22-183	>1000	>1000	>1000	>1000
P.h. var. acuformis	22-116	0.6	ND	ND	2.5
P.aestiva	23-1	0.6	ND	ND	1.3
P.angoides	24-1	0.5	ND	ND	<5.0

Figure 3.1 Dose response of three benzimidazole-resistant isolates to carbendazim (▲), benomyl (△), thiophanate-methyl (□) and thiabendazole (●). Growth of colonies measured after eleven days incubation at 19°C.

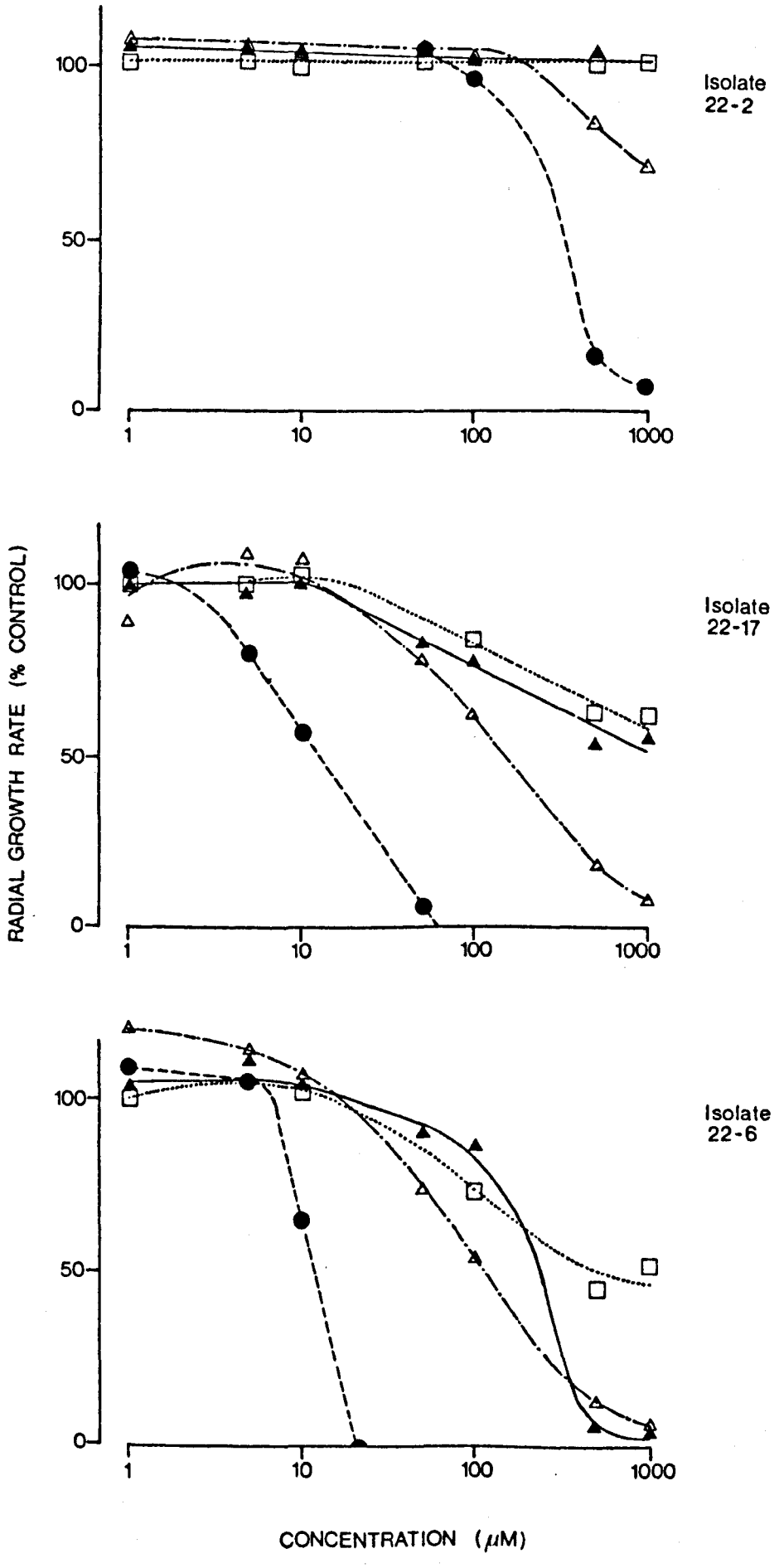
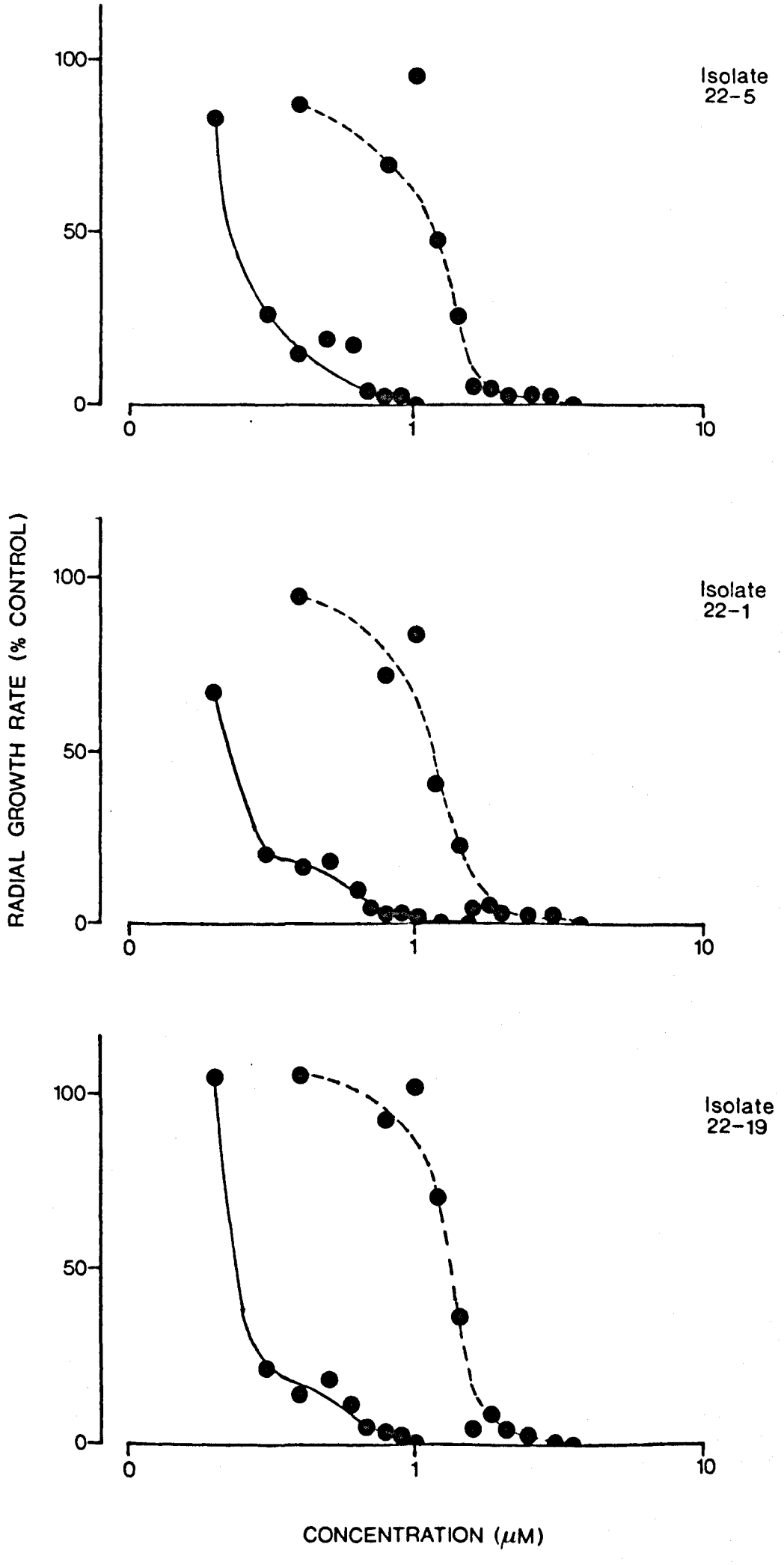

RADIAL GROWTH RATE (% CONTROL)
100
50
0
1
10
100
1000
Isolate 22-2
Isolate 22-17
Isolate 22-6
CONCENTRATION (μM)

Figure 3.2 Dose response of three benzimidazole-sensitive isolates to carbendazim (——) and thiabendazole (---). Growth of colonies measured after eleven days incubation at 19°C.


Isolate
22-5
Isolate
22-1
Isolate
22-19
RADIAL GROWTH RATE (% CONTROL)
100
50
0
0
1
10
CONCENTRATION (μM)

3.3.2 Benzimidazole Sensitivity of Laboratory Mutants

To investigate the expression of benzimidazole resistance against a less variable genetic background than is obtained when comparing field isolates, mutants altered in their response to this group of fungicides were produced from two sensitive isolates: 22-20, a fast growing, BW-type isolate and 22-184, a sporulation mutant derived from 22-12, a slow growing, BWR-type isolate.

Table 3.2 Benzimidazole sensitivity of field isolates: ED_{50} values (μM) for growth inhibition by carbendazim (MBC), benomyl (BEN), thiophanate-methyl (T-M) and thiabendazole (TBZ).

ISOLATE NUMBER	ED_{50} (μM) MBC	BEN	T-M	TBZ
22-1	0.25	ND	ND	1.12
22-3	0.27	ND	ND	1.32
22-5	0.25	ND	ND	1.16
22-12	0.26	ND	ND	1.50
22-19	0.20	ND	ND	1.32
22-6	240	117	520	12
22-8	370	234	>1000	17
22-17	>1000	158	>1000	13
22-10	>1000	295	>1000	224
22-2	<1000	>1000	>1000	340

3.3.2.1 Mutant Isolation

Resistant, mutant strains were produced by selecting either for spontaneous mutants or for induced mutants following UV-irradiation of spores. Eight spontaneous resistance mutants were obtained as fast growing sectors from agar plug inocula on medium containing carbendazim, benomyl or thiophanate-methyl at

concentrations around the MIC for the parent isolate (Table 3.3).

Spontaneous resistance mutants were also isolated by plating conidia on MYG agar supplemented with inhibitory levels of carbendazim or thiabendazole. The mutation frequency (Table 3.4) was very low (4.0×10^{-9}) which was consistent with the findings of previous workers (Fehrmann et al, 1977,1982).

Seventy-one resistance mutants were produced from isolate 22-20 and one hundred andninety-eight from isolate 22-184 after UV-mutagenesis. The induced mutation frequency ranged from 5.0×10^{-5} to 2.1×10^{-6} depending on the strain and the method of selection (Table 3.5).

Table 3.3 Spontaneous resistance mutants derived as sectors from isolate 22-20

SELECTIVE COMPOUND	ISOLATE 22-20 MIC (µM)	ED50 (µM)	SELECTIVE CONCENTRATION (µM)	NUMBER OF MUTANTS OBTAINED
MBC	2.5	0.32	1	1
BEN	5.0	ND	1	2
T-M	10	ND	1	1
			5	1
			10	3

3.3.2.2 Characterisation of resistant mutants

The sensitivity of mutants produced from both 22-20 and 22-184 to carbendazim and thiabendazole was assessed in vitro.

Eighty-three benzimidazole resistant mutants derived from 22-20 were categorised on the basis of carbendazim sensitivity (Table 3.6). Three distinct levels of resistance were observed:

Table 3.4 Spontaneous resistance mutants derived from spores of isolate 22-20

	SELECTION SYSTEM	NUMBER OF MUTANTS OBTAINED	SPONTANEOUS MUTATION FREQUENCY
EXPERIMENT 1	5 µM MBC	2	4×10^{-9}
	5 µM TBZ	2	4×10^{-9}
EXPERIMENT 2	1 µM MBC	0	$<1.1 \times 10^{-9}$
	1.3µM MBC	0	$<1.1 \times 10^{-9}$
	4 µM TBZ	0	$<1.9 \times 10^{-9}$

Table 3.5 Frequency of induced mutation to benzimidazole resistance of spores following UV-irradiation

PARENTAL ISOLATE	SELECTION SYSTEM	EXPOSURE TIME (s)	SURVIVAL (%)	NUMBER OF MUTANTS OBTAINED	INDUCED MUTATION FREQUENCY
22-20	1µM MBC	40	9.1	4	5.0×10^{-5}
	20µM TBZ	40	7.4	3	2.1×10^{-6}
22-184	5µM TBZ	40	42.4	76	9.4×10^{-6}

high-level resistant mutants, which were able to grow even on 1000 µM carbendazim, often with no apparent reduction in growth rate, intermediate-level resistant strains, where mycelial growth was prevented by concentrations of carbendazim above 50 to 200 µM and low-level resistant mutants in which complete inhibition of growth occurred between 5 and 20 µM. Within each of these groupings, variation was found in the precise level of

carbendazim resistance expressed, based on MIC and, to a greater extent, in the levels of resistance to thiabendazole. While all mutants showed cross resistance to both carbendazim and thiabendazole, the degree of resistance to these two fungicides was not directly related (Table 3.6). Generally, however, mutants with a high level of carbendazim resistance were more resistant to thiabendazole than those with low-level carbendazim resistance.

The levels of resistance of one hundred and ninety-eight UV-induced mutants of the BWR-type isolate, 22-184, were similarly determined, and the strains arranged according to sensitivity to carbendazim (Table 3.7). Three resistance categories, equivalent to those identified for the 22-20 mutants, were found and, in addition, a group of strains exhibiting a very low level of carbendazim resistance (MIC = 1.25 μM) but with a higher degree of resistance to thiabendazole. As before, all mutants were cross-resistant to thiabendazole, the level of thiabendazole resistance being quite variable.

The frequency of isolation of particular resistance types from the two parental isolates showed some differences. A far greater proportion of 22-184 mutants were of the low-level carbendazim-resistant type than was obtained from isolate 22-20. This difference is probably due to the use of different selection systems for the isolation of mutants from the two strains. Most resistance mutants from 22-20 were selected with either 10 μM carbendazim or 20 μM thiabendazole, while those derived from 22-

184, because of the greater sensitivity of the parental strain, were isolated on medium containing only 5 μM thiabendazole.

Table 3.6 Resistance expression in mutants derived from isolate 22-20: mutants classified on the basis of the level of resistance to carbendazim (MBC) and thiabendazole (TBZ)

MBC RESISTANCE LEVEL	MIC (μM)		NUMBER OF MUTANTS PER GROUP	TOTAL NUMBER OF MUTANTS
	MBC	TBZ		
SENSITIVE (WILD TYPE)	2.5	3	-	-
LOW	5	5	8	
	10-20	∿20	11	36
	10-20	∿50	10	
	10-20	>100	7	
INTERMEDIATE	50-200	>1000	18	18
HIGH	>1000	∿100	5	
	>1000	>1000	24	29
Total number of mutants screened				83

Table 3.7 Resistance expression in mutants derived from isolate 22-184, classified on the basis of the level of resistance to carbendazim (MBC) and thiabendazole (TBZ).

MBC RESISTANCE LEVEL	MIC (μM) MBC	MIC (μM) TBZ	NUMBER OF MUTANTS PER GROUP	TOTAL NUMBER OF MUTANTS
SENSITIVE (WILD TYPE)	0.6	1.25	-	-
VERY LOW	1.25	10->10 <100	16	16
LOW	5->10 <50	>10 <100	3	
	1.25-10	2,5->10 <100	145	
	10	>100	2	
	>10 <50	>10 <100	6	
	>10 <50	>100	10	166
INTERMEDIATE	<50 <1000	>100	4	4
HIGH	>1000	>100	12	12
Total number of mutants screened				198

3.3.3 N-Phenylcarbamate Sensitivity Relationships

To further characterise the expression of benzimidazole resistance in Pseudocercosporella, the response of field isolates and laboratory resistance mutants to the developmental N-phenylcarbamate fungicides, MDPC and S32165, was compared.

3.3.3.1 N-Phenylcarbamate Response of Field Isolates

Determination of MIC values for MDPC and S32165 to a number of field isolates showed clear differences in sensitivity. Isolates could be identified as either sensitive or insensitive to MDPC and S32165 on the basis of their ability to grow at single diagnostic concentrations of the two compounds (50 and 75 µM, respectively). All fourteen benzimidazole-sensitive field isolates tested were insensitive to MDPC and S32165 (Table 3.8). Of the benzimidazole-resistant field isolates, fifteen were sensitive to MDPC and S32165, while two, 22-7 and 22-11, were sensitive only to MDPC. The type isolates of P. h. var.acuformis, P. auguoides and P. aestiva, like the benzimidazole-sensitive P. herpotrichoides field isolates, were insensitive to the two phenylcarbamates. Complete negatively-correlated cross-resistance was therefore observed between carbendazim and MDPC, in the isolates tested, while the correlation between benzimidazole-resistance and sensitivity to S32165 was not complete.

3.3.3.2 N-Phenylcarbamate Response of Laboratory mutants

Considerable variation in patterns of cross-resistance was found in the induced-resistance mutants (Table 3.9). Of those mutants derived from 22-20, negatively-correlated cross-

resistance was almost exclusively limited to the mutants with high-level carbendazim-resistance. Within this group of twenty-nine mutants four (17%) showed increased sensitivity to both phenylcarbamates, twenty-four(83%) had increased sensitivity only to MDPC and one (~3%) was insensitive to both MDPC and S32165. None of the eighteen intermediate-level resistant mutants and only one of the low-level resistant mutants was altered in its response to these compounds. This single mutant exhibited wild-type sensitivity to S32165 but was sensitive to 50 µM MDPC.

A slightly different pattern was observed with resistant mutants of isolate 22-184. While eight of the twelve high-level resistant mutants had increased MDPC sensitivity, none showed any change in S32165 response. The absence of mutants with increased sensitivity to both compounds may be due to the smaller number of high-level resistant mutants obtained from this isolate. The phenylcarbamate sensitivity of all the intermediate-level resistant strains and most of the low-level resistant mutants was unchanged. The proportion of low-level resistant mutants with increased sensitivity to MDPC was similar to that found in the mutants of 22-20.

However, the main difference between the resistance mutants obtained from isolates 22-20 and 22-184 was the identification of a group of 22-184 derived strains in which increased sensitivity to both the phenylcarbamate compounds was not associated with a high level of carbendazim or thiabendazole resistance. Initially these strains were classified as sensitive to both the phenylcarbamate and benzimidazole fungicides, however, closer examination revealed they were all altered, to a limited extent,

Table 3.8 Negatively-correlated cross-resistance to the N-phenylcarbamate fungicides MDPC and S32165 in field isolates of P. herpotrichoides.

BENZIMIDAZOLE SENSITIVITY	ISOLATE TYPE	ISOLATE NUMBER	MIC (µM) MDPC	S32165
SENSITIVE	BW	22-20	>100	>150
		22-22	>100	>75
		22-21	>100	>150
		22-1	>50	>75
		22-5	>50	>75
		22-18	>50	>75
		22-19	>50	>75
	BWR	22-12	>100	>150
		22-117	>100	>150
		22-120	>100	>150
		22-121	>100	>150
RESISTANT	BW	22-2	20	9
		22-118	10	19
	BWR	22-6	10	<5
		22-10	10	19
		22-11	10	>150
		22-15	10	ND
		22-119	10	9
		22-182	10	38
		22-183	20	9
		22-4	<50	<75
		22-8	<50	<75
		22-9	<50	<75
		22-13	<50	<75
		22-14	<50	<75
		22-16	<50	<75
		22-17	<50	<75
		22-7	<50	>75
SENSITIVE	P.h. var. acuformis	22-116	>100	>150
	P.aestiva	23-1	>100	>150
	P.anguoides	24-1	>50	>75

in sensitivity to carbendazim and thiabendazole, and were accordingly classified as "very low-level" resistance mutants (Table 3.9). Mutants of this type were not described amongst those derived from isolate 22-20 possibly because the resistant mutants from 22-184 were selected on a significantly lower concentration of thiabendazole. Had the same level been used to obtain the 22-184 mutants that was employed in the selection of the 22-20 mutants (20 µM), it is unlikely that this group of mutants would have been recovered.

3.3.3.3 Reversion in MDPC-sensitive mutants

The relationship between carbendazim and thiabendazole sensitivity and MDPC and S32165 sensitivity is obviously complex and may involve mutations in more than one gene. This relationship can be further explored by examining the resistance profiles produced following spontaneous forward mutation to MDPC insensitivity.

Spores from strain 22-49, a high-level carbendazim-resistant mutant derived from isolate 22-20, showing negatively-correlated cross-resistance to MDPC but not S32165, were plated on MYG agar containing 50 µM MDPC. After incubation at 19°C for 14 to 21 days six fast growing and twelve slow growing, apparently resistant colonies were obtained. When screened for sensitivity to carbendazim, thiabendazole, MDPC and S32165 five distinct groups could be recognised. The first group were apparently direct back-mutations, exhibiting the wild-type benzimidazole sensitivity and concomitant phenylcarbamate insensitivity. The second group retained the high level

Table 3.9 N-Phenylcarbamate sensitivity of benzimidazole-resistant mutants derived from isolate 22-20 and 22-184

PARENTAL ISOLATE	MBC RESISTANCE LEVEL	MIC (µM) MDPC	MIC (µM) S32165	NUMBER OF MUTANTS PER GROUP
22-20 BW-TYPE	SENSITIVE	>100	>150	
	LOW	<50	>75	1
		>50	>75	35
	INTERMEDIATE	>100	>150	18
	HIGH	>5 <10	<5-9	4
		>20 <50	>150	24
		>100>	150	1
22-184 BWR-TYPE	SENSITIVE	>100	>150	
	VERY LOW	<50	<0.6	16
	LOW	<50	>150	3
		>50	>150	163
	INTERMEDIATE	>50	>150	4
	HIGH	<50	>150	8
		>50	>150	4

carbendazim resistance of the parental strain, had increased resistance to thiabendazole, being able to grow on 1000 µM, and showed decreased MDPC-sensitivity, being able to grow at 50 µM but not at 100 µM MDPC. Group three strains also retained the carbendazim resistance of the parent and showed a smaller, but significant increase in thiabendazole resistance. Sensitivity to

MDPC appeared unchanged. The fourth group had the benzimidazole resistance levels of the resistant parent and paradoxically showed slightly increased sensitivity to MDPC, the degree of growth inhibition observed at 2.5 to 10 µM MDPC being greater than for the parent strain. The fifth and last group were altered in their response to carbendazim. Mycelial growth, while still produced at 1000µM, was inhibited at concentrations above 50µM carbendazim. Thiabendazole resistance was slightly increased compared with 22-49, but MDPC sensitivity was apparently unaltered.

The carbendazim-resistant parental strain, 22-49, was insensitive to S32165. None of the mutants produced on MDPC showed any increase in sensitivity to this compound. Table 3.10 summarises the fungicide-sensitivity profiles of these mutants.

3.3.4 Sensitivity to Ergosterol Biosynthesis Inhibitors

Dose-response data for a number of field and type isolates to various EBI fungicides was used to calculate the concentrations of each fungicide required to reduce mycelial growth by 50, 75 and 90%; the isolates were then ranked according to sensitivity to the compounds (Table 3.11).

Clear differences in sensitivity were seen between BW and BWR pathotypes, particularly in response to the triazole derivatives triadimenol, propiconazole, and DPX H6573. The BWR-type isolates were generally much less sensitive to these fungicides than were BW-types. In contrast, most BWR-type isolates were more sensitive to the morpholine fungicide, fenpropimorph, although this correlation was not absolute. Sensitivity to prochloraz, an

Table 3.10 Fungicide sensitivity profiles of strains derived from the MBC high-level resistance mutant 22-49, by selection on 50 µM MDPC. Minimum inhibitory concentrations assessed after 10 days at 19°C.

	MIC (µM)				NUMBER OF MUTANTS PER GROUP
	MBC	TBZ	MDPC	S32165	
22-20 WILD TYPE	2.5	5	>100	>150	
22-49 RESISTANT MUTANT	>1000	>20 <50	>20 <50	>150	
GROUP 1	2.5	5	>100	>150	3
GROUP 2	>1000	>1000	>50 <100	>150	3
GROUP 3	>1000	100-1000	>20 <50	>150	7
GROUP 4	>1000	>20 <50	∿20	>150	3
GROUP 5	>1000*	∿100	>20 <50	>150	2

*growth rate significantly reduced at concentrations above 50 µM.

imidazole compound, was much less variable. However, comparison of growth inhibition at 0.2 µM prochloraz suggested that BW isolates may be slightly less sensitive than BWR-types. BW isolate 22-21 and to a lesser extent 22-22, were frequently anomalous in their response to these compounds. Isolate 22-21 was originally isolated from oats and had a peculiar morphology, while isolate 22-22 was isolated from rye as a BW strain with very profuse aerial mycelium. Their responses to propiconazole, fenpropimorph and to a lesser degree triadimenol and DPX H6573, were more characteristic of the BWR-types tested.

Sensitivity of the P. anguoides and P. aestiva type-isolates (24-1 and 23-1 respectively) was different to that of either of the two P. herpotrichoides pathotypes. The former were reasonably sensitive to both triadimenol and fenpropimorph (like 22-21 and 22-22) but differed considerably in their responses to propiconazole, DPX H6573 and prochloraz. The sensitivity of P. anguoides to this last compound was similar to most of the BWR-types, while P. aestiva was relatively insensitive to prochloraz, 50% growth inhibition not being attained even at 40 µM, the highest concentration tested in this experiment.

No correlation was found between reduced sensitivity to EBI fungicides and resistance to benzimidazoles, either in field isolates or in carbendazim-resistant mutants derived from isolate 22-184 (Table 3.12).

TRIADIMENOL				PROPICONAZOLE				DPX H6573				FENPROPIMORPH				PROCHLORAZ				Growth rate
Isolate number	ED_{50}	ED_{75}	ED_{90}	Isolate number	ED_{50}	ED_{75}	ED_{90}	Isolate number	ED_{50}	ED_{75}	ED_{90}	Isolate number	ED_{50}	ED_{75}	ED_{90}	Isolate number	ED_{50}	ED_{75}	ED_{90}	on 0.2 µM (% control)
22-116	>400	>400	>400	22-120	6.9	9.8	10.8	23-1	0.9	14.8	21.9	22-12	53	160	ND	23-1	>40	>40	>40	58.2
22-121	383	>400	>400	22-119	5.0	10.2	13.2	22-21	1.3	6.6	11.2	22-119	31	135	ND	22-18	0.3	0.6	0.8	56.5
22-120	310	>400	>400	22-4	4.1	8.1	10.2	22-4	0.7	1.7	2.7	22-18	17	114	ND	22-21	<0.2	0.4	1.0	35.1
22-119	250	>400	>400	22-21	2.6	12.9	33.1	22-119	0.6	1.1	1.4	22-4	15	114	>200	22-1	<0.2	0.3	0.8	36.7
22-183	211	>400	>400	22-116	3.4	6.7	10.2	22.7	0.4	1.0	1.6	22-5	<10	89	>200	22-5	<0.2	0.3	0.5	34.1
22-6	192	>400	>400	23-1	2.4	6.8	11.7	22-116	0.5	0.8	1.4	22-1	17	49	180	22-4	<0.2	0.3	0.5	38.8
22-8	178	>400	>400	22-121	2.4	6.3	8.9	22-9	0.3	1.3	1.6	22-9	11	100	155	22-183	<0.2	0.2	0.6	26.7
22-117	100-200	>400	>400	22-6	1.8	4.6	8.3	22-15	0.3	1.2	1.6	22-17	16	68	138	22-2	<0.2	0.2	0.3	25.9
22-16	107	>400	>400	22-15	1.8	4.3	7.2	24-1	0.4	0.9	1.4	22-118	19	62	115	22-118	<0.2	<0.2	0.3	21.9
22-15	102	>400	>400	22-7	1.3	4.4	8.3	22-183	0.4	0.9	1.2	22-2	18	54	104	22-20	<0.2	<0.2	ND	19.1
22-7	58	369	>400	22-8	1.2	4.1	7.4	22-120	0.4	0.6	0.8	22-20	21	45	93	22-17	<0.2	<0.2	ND	16.8
22-22	49	282	>400	22-183	1.2	3.8	7.4	22-3	0.3	0.6	0.9	22-116	<10	63	ND	22-7	<0.2	<0.2	ND	12.8
22-21	32	108	224	22-1	0.9	2.4	3.8	22-12	0.3	0.6	0.9	22-8	<10	39	140	22-9	<0.2	<0.2	0.2	12.2
24-1	16	46	66	22-22	0.9	1.8	3.4	22-16	0.3	0.4	ND	22-15	<10	39	63	22-16	<0.2	<0.2	<0.2	7.7
23-1	10	46	112	22-16	0.9	1.7	2.5	22-10	0.2	0.5	0.9	22-7	<10	30	72	22-22	<0.2	<0.2	<0.2	6.8
22-1	10	19	27	24-1	0.7	1.3	2.6	22-22	<0.2	0.5	0.8	22-16	<10	30	66	22-119	<0.2	<0.2	<0.2	6.6
22-2	6	9	12	22-2	0.5	0.9	1.1	22-1	<0.2	0.5	0.7	23-1	<10	28	65	24-1	<0.2	<0.2	<0.2	6.5
22-118	<5	8	11	22-18	0.4	1.0	1.9	22-18	<0.2	ND	ND	22-183	<10	16	45	22-116	<0.3	<0.2	<0.2	4.4
22-20	<5	7	9	22-20	0.3	0.7	1.1	22-121	<0.2	0.3	0.4	22-10	<10	13	45	22-3	<0.2	<0.2	<0.2	4.3
								22-2	<0.2	<0.2	0.4	22-3	<10	21	30	22-121	<0.2	<0.2	<0.2	2.0
								22.20	<0.2	<0.2	0.3	22-117	<10	16	ND	22-15	<0.2	<0.2	<0.2	1.8
								22-118	<0.2	<0.2	ND	22-120	<10	13	20	22-120	<0.2	<0.2	<0.2	0.4
								22-5	<0.2	ND	ND	22-22	<10	10	ND	22-10	<0.2	<0.2	<0.2	0
								22-17	<0.2	<0.2	ND	22-21	<10	<10	<10	22-12	<0.2	<0.2	<0.2	0
								22-117	<0.2	ND	ND	24-1	<10	<10	<10	22-8	<0.2	<0.2	<0.2	0
												22-121	<10	<10	<10	22-117	<0.2	<0.2	<0.2	0

Table 3.11. Response of Pseudocercosporella field and type isolates to five ergosterol biosynthesis inhibiting fungicides. ED values expressed in µM and ranked in order of increasing sensitivity. P. herpotrichoides BWR-type isolates shown in red, BW-type isolates in blue. P. aestiva (23-1) and P. auguoides (24-1) shown in green. (ND = not determined).

Table 3.12 Sensitivity to three ergosterol biosynthesis inhibiting fungicides of mutants, derived from the BWR-type isolate 22-184, altered in their reponse to benzimidazole and phenylbarbamate fungicides.

ISOLATE NUMBER	BENZIMIDAZOLE/PHENYLCARBAMATE SENSITIVITIES (M.I.C.s in µM)				TRIADIMENOL SENSITIVITY (µM)			PROPICONAZOLE SENSITIVITY (µM)			UK 200 SENSITIVITY (µM)		
	MBC	TBZ	MDPC	S32165	ED50	ED75	ED90	ED50	ED75	ED90	ED50	ED75	ED90
22-184 (wild type)	0.6	1.25	>50	>150	72	304	ND	0.6	2.0	4.0	<5	17	68
22-235	1.25	10	50	<0.6	64	251	ND	0.4	1.1	2.9	<5	16	21
22-241	1.25	10	50	<0.6	98	293	ND	0.9	1.6	2.5	<5	7	33
22-239	>1000	>100	50	>150	98	196	ND	0.6	1.0	1.7	<5	10	21
22 240	>1000	>100	>50	>150	131	414	ND	1.0	1.6	2.1	5	12	19

ND = no data

3.4 DISCUSSION

The occurrence of benzimidazole resistance in *P. herpotrichoides* is well documented (Brown *et al*, 1984; King & Griffin, 1985). In most published reports resistant isolates were capable of growth in the presence of extremely high concentrations of the fungicide (100 to 1000 $\mu g\ ml^{-1}$). Similarly all 17 carbendazim-resistant isolates tested in this study were able to produce mycelial growth at 1000 µM carbendazim. However, there was some variation in the dose-responses between resistant isolates. In all cases resistance to carbendazim was associated with resistance to the other fungicides in this group. Cross-resistance to benomyl and thiophanate-methyl was anticipated, since both these compounds generate carbendazim as the active component in aqueous solution (Clemons & Sisler, 1969; Courtney, 1977). Differences in response to benomyl and thiophanate-methyl can be related to the rate of conversion to carbendazim of these compounds and to the production of the fungitoxic by-product, butyl isocyanate, during the hydrolysis of benomyl (Hammerschlag & Sisler, 1972, 1973).

Thiabendazole, however, is not converted into carbendazim but is fungitoxic in its original state (Davidse & Flach, 1978). Consequently, while cross-resistance to this compound is usual, the level of resistance expressed is not necessarily related to resistance to carbendazim. The variation in carbendazim and thiabendazole resistance observed in field isolates suggests that benzimidazole-resistance has arisen separately in this pathogen on several occasions, and that fungicide resistant isolates are

not clones of one progenitor strain.

Analysis of induced mutants revealed three main recognisable levels of carbendazim resistance. In each case cross-resistance to thiabendazole was observed, although the levels of thiabendazole resistance were variable with many low-level carbendazim-resistant strains growing on concentrations of thiabendazole in excess of 100μM. In general, however, the higher the level of carbendazim resistance the greater the level of resistance to thiabendazole.

The level of carbendazim-resistance observed in most field isolates was equivalent to that of the high-level resistance mutants produced in the laboratory. No reports have been published of strains exhibiting low-level carbendazim resistance being isolated from the field. However, Brown et al (1984) did describe one resistant isolate of P. herpotrichoides which grew on carbendazim concentrations of up to 50 μg ml^{-1} (~260 μM) but was inhibited at higher doses. This isolate would appear similar in response to mutants classified as showing intermediate level carbendazim resistance in this work. The absence of P. herpotrichoides isolates from field surveys with different levels of carbendazim resistance can be related, at least in part, to the methods used to screen field populations for resistance to fungicides. Generally isolates are tested on only a single concentration of the fungicide, chosen because it is significantly greater than the highest dose tolerated by sensitive isolates (Bateman et al, 1985; King & Griffin, 1985). As a result not only are strains with low-level resistance unlikely to be identified but the level of resistance shown by

resistance expression following mutagenesis. Similarly spontaneous resistant sectors were obtained more readily using thiophanate-methyl as the selective agent than with the more fungitoxic carbendazim.

Negatively-correlated cross-resistance between benzimidazoles and the developmental N-phenylcarbamate fungicides, MDPC and S32165, was found in both field isolates and laboratory mutants. All the carbendazim-resistant field isolates exhibited increased sensitivity to MDPC, while fourteen of the sixteen tested were extra-sensitive to S32165. The remaining two isolates were insensitive to S32165 while showing increased sensitivity to MDPC.

The patterns of cross-resistance were more complicated for the laboratory produced mutants. In most cases increased phenylcarbamate sensitivity was only shown by high-level carbendazim-resistant mutants, intermediate- and low-level carbendazim-resistance usually being associated with wild-type phenylcarbamate sensitivity. However, not all the high-level resistant mutants exhibited negative-cross resistance, some were insensitive to S32165 and some were not inhibited by either compound. In addition four low-level resistance mutants, three derived from 22-184 and one from 22-20, had increased MDPC sensitivity, although to a lesser degree than that found in high-level carbendazim-resistant mutants.

The occurrence of strains with significant levels of benzimidazole resistance without altered phenylcarbamate sensitivity has obvious implications for the use of mixtures of these compounds to control carbendazim-resistant P.

herpotrichoides strains in the field, especially since the detection of naturally occurring S32165-insensitive, carbendazim-resistant isolates strongly suggests that this phenotype is not associated with reduced fitness. Furthermore, the ease with which strains exhibiting reduced MDPC sensitivity, while retaining high-level carbendazim resistance, can be produced in the laboratory indicates that phenylcarbamate-insensitive strains may be selected within the present benzimidazole-resistant field population by using these compounds.

The identification of so many resistance/sensitivity phenotypes in P. herpotrichoides, including a group in which increased phenylcarbamate sensitivity is associated with a level of carbendazim sensitivity only slightly lower than the wild-type, raises a number of important questions:

1) are the different levels of resistance to carbendazim due to changes in the same gene?

2) is phenylcarbamate sensitivity and bendimidazole resistance controlled by the same gene(s)?

3) is the mechanism of resistance in the various mutant types identical?

4) if resistance is controlled by a number of different genes, do they interact?

Some insight into these questions may be derived by comparison with other fungal species.

Allelic variation in the level of benzimidazole resistance has been demonstrated in Venturia inaequalis (Katan et al, 1983; Shabi et al, 1983; Stanis & Jones, 1984), V. nashicola

(Ishii & Yanase, 1983), V. pirina (Shabi et al, 1986), Ustilago maydis, Aspergillus nidulans (benA mutants) and A. niger (van Tuyl, 1977), Neurospora crassa (Borck & Braymer, 1974), Talaromyces flavus (Katan et al, 1984) and Ceratocystis ulmi (Webber et al, 1986). In several instances however, two or more interacting genes have been implicated in the expression of benzimidazole resistance. In A. nidulans, the most characterised species, two loci in addition to benA, were found to confer resistance to this group of fungicides. In each case the level of resistance was uniformly low, while mutations in benA produced a range of resistance phenotypes from low to very high. Additive effects on resistance expression were found with combinations of benA with benB or benC but no increase in resistance occurred when benB and benC were together.

In Shizosaccharomyces pombe three genes were found to be involved in benzimidazole resistance expression. One (ben-1) conferred high-level, temperature-dependant resistance while the other two (ben-2 and ben-3) produced low-level resistance which was decreased at lower temperatures (Yamamoto, 1980; Yamamoto & Sakaguchi, 1982). Beraha and Garber (1980) showed that the development of intermediate- and high-level resistance to thiabendazole in Penicillium italicum occurred only in strains that had previously acquired low-level resistance and produced evidence for a system involving two or three closely linked genes controlling the expression of this resistance. A two gene system was recently described controlling benzimidazole resistance in Fusarium oxysporum (Molnar et al, 1986). Higher levels of benomyl resistance were produced when the two mutant genes, ben-1 and

ben-2, were both present. Separately they conferred only a moderate level of resistance. Polygenic control of benzimidazole resistance has also been described, in Ustilago hordei (Ben-Yephet et al, 1975).

Observations on the expression of negatively-correlated cross-resistance between benzimidazole and phenylcarbamate fungicides, similar to those for P. herpotrichoides have been made for V. nashicola (Ishii et al, 1984). In this species three levels of carbendazim resistance were identified. Increased sensitivity to MDPC was associated only with the highest level of resistance. Similarly in V. pirina MDPC extra-sensitivity was restricted to those isolates exhibiting very high-level resistance to benzimidazoles (Shabi et al, 1986).

Leroux et al (1985a) classified field isolates of P. herpotrichoides into four groups on the basis of benzimidazole and barban (4-chloro-2-yl-3-chlorophenylcarbamate) sensitivity. The first group (A) were benzimidazole sensitive isolates which were relatively insensitive to barban. The ED_{50} for mycelial growth of these isolates was 0.06 mg ml^{-1} (0.31 µM) for carbendazim and 0.06 mg ml^{-1} (1.0 µM) for thiabendazole, which agree closely with the values produced for sensitive isolates in this work. The second group (B) consisted of resistant isolates with ED_{50} values for carbendazim and thiabendazole of 60 mg ml^{-1} (314 µM) and 2 mg ml^{-1} (10 µM) respectively. These isolates showed increased sensitivity to barban. Isolates 22-6 and 22-8 in this study (Table 3.2) appear to of the same type with respect to their response to benzimidazoles and both showed negatively-

correlated cross-resistance to MDPC and S32165. The third group (C) were also extra sensitive to barban and had ED_{50} values in excess of 100 mg ml^{-1} (>523 μM) for carbendazim. The ED_{50} for thiabendazole for these strains was 40 mg ml^{-1} (199 μM). These isolates are similar to 22-2 and 22-10 studied here. The final group (D) were highly resistant to the benzimidazoles, the ED_{50} for carbendazim was greater than 100 mg ml^{-1} (>523 μM) and for thiabendazole was 8 mg ml^{-1} (40 μM), but did not show increased sensitivity to barban. It would be of interest to compare these isolates, described as being rare in nature, with field isolates studied in this work which were highly benzimidazole-resistant but insensitive to S32165 (22-7 and 22-11, Table 3.8).

Resistance to the benzimidazole fungicides in different species has been shown to have more than one basis. Mechanisms include reduced uptake of the fungicide (Nachmias & Barash, 1976; Tripathi & Schlosser, 1982; Welker & Williams, 1983) and increased extracellular acid production (Lambert & Wuerst, 1976). The main mechanism of resistance to these compounds, however, appears to involve modification of the site of action for these fungicides, the β-tubulin protein (Davidse & Flach, 1977, 1978; Sheir-Neiss et al, 1978; Tripathi & Schlosser, 1982; Neff et al, 1983; Orbach et al, 1986; Roobal et al, 1984).

Van Tuyl (1977a) showed by crossing mutants with differing resistance phenotypes that recombination within the A. nidulans benA gene was possible, demonstrating that mutations at a number of points in this gene can result in altered fungicide response. By analogy it is quite possible that mutations at a number of points in one or more areas of the β-tubulin structural gene in

P. herpotrichoides could result in the diverse range of resistance phenotypes observed.

Alternatively a proportion, or indeed all, these resistance types may be the result of mutation in genes other than those coding for β-tubulin. In S. pombe, in addition to mutants for the β-tubulin structural gene (nda-3), altered sensitivity to benzimidazole compounds was found to result from changes in α-tubulin, caused by mutation in the structural gene for this protein (nda-2) (Toda et al, 1984; Hiraoka et al, 1984). The mechanism of action of the resistance controlled by the benB and benC loci in A. nidulans has not yet been clarified, but does not seem to involve the tubulin proteins. Furthermore, the basis of resistance of several of the benzimidazole insensitive mutants of Dictyostelium discoideum studied by Welker & Williams (1983) was assumed to involve altered membrane permeability since these strains were cross-resistant for the unrelated toxicants methanol and acriflavine.

Reversion to MDPC insensitivity may be due either to back mutation or to intra- or extragenic suppressors of sensitivity. If carbendazim resistance and MDPC extra-sensitivity is due to the same mutation, MDPC-insensitive revertants, caused by back mutation, would be expected to result in the loss of carbendazim-resistance. This was indeed the case with three of the eighteen revertants studied. The remaining mutants presumably carry supressor mutations. The presence of supressors did not necessarily lead to reduced benzimidazole resistance, in some cases thiabendazole resistance was actually increased.

Supressor mutations associated with the expression of the benA gene have been extensively studied in A. nidulans (Morris et al, 1979; Oakley et al, 1985). benA mutants in which resistance to benzimidazoles was associated with heat sensitivity were used to select for temperature-insensitive revertants. Analyses of these revertant strains showed that a proportion were due to back-mutation in the benA gene resulting in concomitant loss of benzimidazole resistance. Others carried extragenic suppressors some of which also caused a loss of resistance and cold sensitivity. One extragenic suppressor was later identified as the structural gene for α-tubulin, tubA (Morris et al, 1979). Intragenic suppressors of heat sensitivity, selected in a benA benomyl-resistant mutant often caused cold sensitivity and, at least in one case, loss of resistance. Most revertants, however, retained a significant degree of benomyl resistance.

Seventeen of these intragenic suppressors were subjected to fine structure mapping and shown to be closely linked to the original mutation. Eleven of these mutations were clustered at two sites which it was thought, represented regions of particular importance for the functioning of the β-tubulin protein in this mutant. (Oakley et al, 1985). These studies clearly demonstrate the capacity for variation in the tubulin genes and the diversity of associated resistance phenotypes which can be produced.

The variation in sensitivity to various inhibitors of ergosterol biosynthesis among P. herpotrichoides field isolates is disturbing, since it implies that particular pathotypes may be indirectly selected in the field by the use of these compounds to control unrelated fungal disease agents. Surveys of P.

herpotrichoides field populations, carried out at ADAS (Bristol), have demonstrated an association between the use of EBI fungicides (excluding morpholines) and the preponderance of BWR pathotypes, particularly in winter barley (Griffin, 1985). This effect was confirmed by Leroux and Gredt (1985a). Wheat plants inoculated with BW- or BWR-type strains and treated with triadimenol, had 20% and 93% infection respectively when assessed after two months.

In the present work resistance to triadimenol was correlated with reduced sensitivity to the other triazole compounds, propiconazole and DPX H6573. An additional, experimental, triazole compound, supplied by Bayer UK Ltd., produced similar results (Data withheld by request). Leroux and Gredt (1985a, b) and Leroux et al (1985) found a similar relationship in triadimenol-insensitive isolates of the fungus from France, which also showed decreased sensitivity to the triazoles bitertanol, dichlobutrazole, propiconazole and triadimefon and also to the pyrimidine derivative fenarimol and the imidazole compounds imazalil and penconazole. Cross-resistance between EBI fungicides which inhibit the C-14 demethylation step in sterol biosynthesis has frequently been reported in various species (de Waard & Gieskes, 1977; de Waard et al, 1982; Hollomon, 1982; Schepers, 1983; Stanis & Jones, 1985).

Data produced at ADAS (Bristol) suggested that carbendazim resistance may be associated in BWR-types with reduced sensitivity to several EBI fungicides (Griffin, 1985). In this

study however, while the number of isolates was relatively small, no obvious correlation between carbendazim-sensitivity and response to the EBI compounds was seen. Similarly, comparison of the EBI sensitivities of four benzimidazole-resistant mutants, derived from the BWR-type isolate 22-184, failed to provide clear evidence for this association.

Sensitivity to fenpropimorph was negatively correlated with triazole sensitivity for most isolates of P. herpotrichoides screened. The relationship was not absolute however, isolate 22-119 for example, was highly resistant to both fenpropimorph and the triazoles studied. Leroux and Gredt (1985a, b) described the same phenomenon: most BW isolates were sensitive to triadimenol and other inhibitors of C-14 demethylation, and were also sensitive to fenpropimorph and fenpropidine, which act by inhibiting the C-14 reductase and/or the Δ-8-Δ-7 isomerase stages in sterol synthesis (Barug & Kerkenaar, 1984). BWR isolates were resistant to triadimenol and compounds of similar mode of action and showed increased sensitivity to the morpholines. Several BW-type isolates were less sensitive to triadimenol and were classified into two groups according to their degree of insensitivity. None were as resistant to triadimenol as the BWR strains and they did not show negatively-correlated cross-resistance to fenpropimorph. These strains appear similar in EBI response to the two BW isolates, 22-21 and 22-22, with reduced sensitivity to triadimenol identified in this study. Both these strains, however, were highly sensitive to fenpropimorph.

Negatively-correlated cross-resistance between morpholines and the other EBI fungicides has been reported in

fenarimol-resistant mutants of *P. italicum* (de Waard *et al*, 1982), while mutants of *U. maydis* resistant to inhibitors of C-14 demethylation have been isolated which variously show negative, positive and a lack of cross-resistance to the morpholines (Barug & Kerkenaar, 1984).

The results obtained in this study confirm the findings of Leroux and Gredt (1985a) that insensitivity to triadimenol and other C-14 demethylation inhibitors does not confer cross-resistance to prochloraz in *P. herpotrichoides*. Similarly Brown *et al* (1984) found no difference in prochloraz response between the two pathotypes or between carbendazim-sensitive or -resistant isolates. By contrast, *Erysiphe graminis* isolates insensitive to triadimenol were also resistant to prochloraz (Butters *et al*, 1984). The suggestion that BW isolates were slightly less sensitive to prochloraz is supported by the data of Leroux and Gredt (1985a) who found that the ED_{50} for prochloraz of BW isolates was between 0.06 and 0.07 $\mu g\ ml^{-1}$ (0.16-0.19 μM) while that of BWR-types was 0.03 $\mu g\ ml^{-1}$ (0.08 μM). These differences are small and unlikely to have any effect at the level of the field population. The relative insensitivity of *P. aestiva* to prochloraz has not previously been reported.

While resistance to EBI fungicides has often been described, there are few instances in which the basis of resistance has been determined. Decreased uptake, associated with an energy-dependant efflux mechanism has been shown to be the basis of resistance to fenarimol in *A. nidulans* and *P. italicum* (de Waard & van Nistlerooy, 1979, 1980, 1984). Differential

sensitivity to triadimefon in Cladosporium cucumerinum and Stemphylium radicinum has been attributed to variation in the conversion of this compound into triadimenol, the active form (Gasztonyi & Josepovits, 1984; Fuchs & de Vries, 1984). Metabolism of triadimefon can result in the formation of two diastereomeric forms of triadimenol. The relative proportions of these produced and their relative toxicity to the transforming fungus determines the sensitivity of the fungus to triadimefon (Deas et al, 1984, 1986). This mechanism may also be the basis of triademefon and triademenol sensitivity in P. italicum (Fuchs et al, 1984), but it is unlikely to be the cause of EBI resistance in P. herpotrichoides since it would not result in cross-resistance for the other EBI compounds. P. italicum is uniformly sensitive to the remaining C-14 demethylation inhibiting fungicides (Fuchs et al, 1984).

Resistance to the EBI group of fungicides was considered unlikely to cause problems for disease control in practice because resistant mutants generally were less fit or had reduced pathogenicity (Fuchs et al, 1977; van Tuyl, 1977a; Fuchs & de Waard, 1982; de Waard & Fuchs, 1982). Whatever its cause, EBI insensitivity in P. herpotrichoides was not associated with reduced pathogenicity since BWR-types often predominate in the pathogen population. Resistance to various EBI compounds not associated with reduced fitness or pathogenicity has been described in P. italicum (de Waard et al, 1982), V. inaequalis (Stanis & Jones, 1985) and E. graminis f.sp. hordei (Fletcher & Wolfe, 1981; Butters et al, 1984)

The occurrence of negative cross-resistance between

triadimenol and fenpropimorph in P. herpotrichoides, raises the possibility of using the latter compound to control, specifically, the BWR pathotype in the field. This strategy is unlikely to be successful, however, in view of the existence of strains with reduced sensitivity to both types of fungicide. On the other hand it may be possible that other morpholine derivatives can be synthesised that are more effective than fenpropimorph. The discovery of negatively-correlated cross-resistance to dodine in fenarimol-resistant strains of A. nidulans, C. cucumerinum, P. expansum, P. italicum and U. maydis (de Waard & van Nistelrooy, 1983) suggests that other unrelated compounds may yet prove useful as control agents, specifically of fungicide-resistant strains.

CHAPTER 4

MUTAGENESIS, ISOLATION AND CHARACTERISATION OF MUTANTS

4.1 INTRODUCTION

Readily recognisable strain markers are an obvious prerequisite both for genetic investigations and studies of population dynamics. In order to demonstrate and follow the events occurring in a parasexual cycle in P. herpotrichoides, the introduction of mutations in strains of the fungus was necessary. Auxotrophic mutations have been most widely used for this purpose in other fungi, since they permit the positive selection of any "hybrid" types by their ability to grow on a selective minimal medium. However, although exceptions have been reported, principally in Verticillium albo-atrum (Clarkson & Heale, 1985a,b) and in Venturia inaequalis, where the addition of the appropriate amino-acid or vitamin restored pathogenicity in auxotrophic strains (Wood, 1987), such mutations are generally deleterious for pathogenicity and consequently of little value in experiments involving colonisation of host plants by the fungus. Pigmentation and morphological mutants may be more appropriate for such studies since these mutations are possibly less likely to affect pathogenicity. Many of these markers have another important use in studies of the population dynamics of plant pathogens. The existence of identifiable markers in a particular strain make it possible to follow its development and performance as part of a "field" population. This chapter descibes the isolation of mutants with auxotrophic, fungicide resistance, pigmentation and morphological phenotypes following mutagenic

treatment of conidia from wild-type isolates of P. herpotrichoides.

4.2 MATERIALS AND METHODS

Mutants were obtained following UV-irradiation of conidia. Spore suspensions (10^5 to 10^6 spores ml^{-1}) were exposed to far UV-light from a Philips Germicidal Lamp (2.4 J m^{-2} s^{-1}) for up to 120 s (288 J m^{-2}) with gentle stirring. Initial spore viability and survival after irradiation were estimated by plating dilutions of the spore suspensions onto MYG agar or the appropriate recovery medium.

To determine the numbers of nuclei in conidia, spores were fixed in 70% ethanol, stained with DAPI overnight at 4°C and viewed under UV-light (Vickers M17 Type C Fluorescence microscope.). The number of germ-tubes produced by germinating conidia was also determined. Spores from each irradiation period were allowed to germinate on sterile cellophane disks over TWA for 2 to 6 days at 19°C. Sections of the cellophane were excised, placed on glass slides and examined microscopically. Approximately 150 to 200 germinating spores were counted for each determination.

Resistance mutants were obtained by direct selection. Irradiated spores were plated onto MYG agar containing an inhibitory concentration of either carbendazim or thiabendazole (5 - 20 μM). Colonies appearing after 16 days at 19°C were purified as described and tested for altered fungicide response. Auxotrophic mutants were identified by transferring mycelium from

colonies growing on MYG onto MM. Strains failing to grow appreciably on MM were assumed to be auxotrophic. Nutritional requirements were identified by the method of Holliday (1956). Auxotrophic requirements which could not be determined by this procedure were identified using a modification of the method described for Streptomyces by Hopwood et al (1985). The system of diagnostic plates used for the identification of most of the auxotrophic mutants obtained is given in Appendix III.

Various techniques were used in an attempt to increase the frequency of isolation of auxotrophic mutants. Filtration enrichment for auxotrophic mutants was performed as follows: irradiated conidia were pre-germinated in liquid MM for 3 days at 19 ^{o}C and then filtered through either 4 layers of sterile muslin or lightly packed glass wool. Spores in the filtrate were plated on MYG agar and a sample of the resulting colonies tested for auxotrophy. The effect of liquid holding on the frequency of mutant isolation was also assessed. Spores were held in sterile distilled water for 24 to 72 h at 19^{o}C after mutagenesis before plating onto the recovery medium. The viability of spores was assessed at each stage, and the number of mutants obtained determined either by screening for auxotrophic mutants or by selection for resistance to thiabendazole (5 μM).

Auxotrophic mutants with the same nutritional requirement were tested for complementation by co-inoculation on MM. In this way most of the auxotrophic mutants could be classified into complementation groups. Auxotrophic mutants were further characterised by assessment of growth on MM supplemented with precursors in the mutant biosynthetic pathway. The enzyme assay

used to detect ornithine carbamyl transferase activity in the arginine-requiring mutant, arg-3, was adapted from that described by Barthelmess et al (1974).

4.3 RESULTS

4.3.1 Survival and Mutation Frequency

Spore survival following exposure to UV-light is shown in Figure 4.1. UV doses in excess of 160 J m^{-2} reduced spore survival to below 1%. A shoulder in the curve at lower doses (0 to 48 J m^{-2}), characteristic of multinucleate propagules, was apparent. At higher doses spore viability was reduced logarithmically proportional to the irradiation dose. While Figure 4.1 represents this data from a single experiment, a similar pattern of spore survival was obtained in repeat experiments, although the precise levels of viability at each irradiation dose were found to vary (cf. Table 4.2). Non-irradiated conidia produced between 1 and 5 germ-tubes (mean = 2.6), mainly from the end cells. Following UV-irradiation, the number of germ-tubes formed was considerably reduced, in parallel with decreasing survival (Table 4.1). Furthermore the germ-tubes were produced randomly, no longer principally from the apical cells, suggesting that the remaining cells in these conidia were non-viable.

The frequency of induced mutation at each irradiation period was assessed by determination of the number of mutants produced resistant to the benzimidazole fungicides carbendazim or thiabendazole (Table 4.2). The highest mutation rates were

Figure 4.1 Survival of conidia of P. herpotrichoides isolate 22-20 after exposure to far UV-light (Philips Germicidal Lamp; 2.4 J m^{-2} s^{-1}).

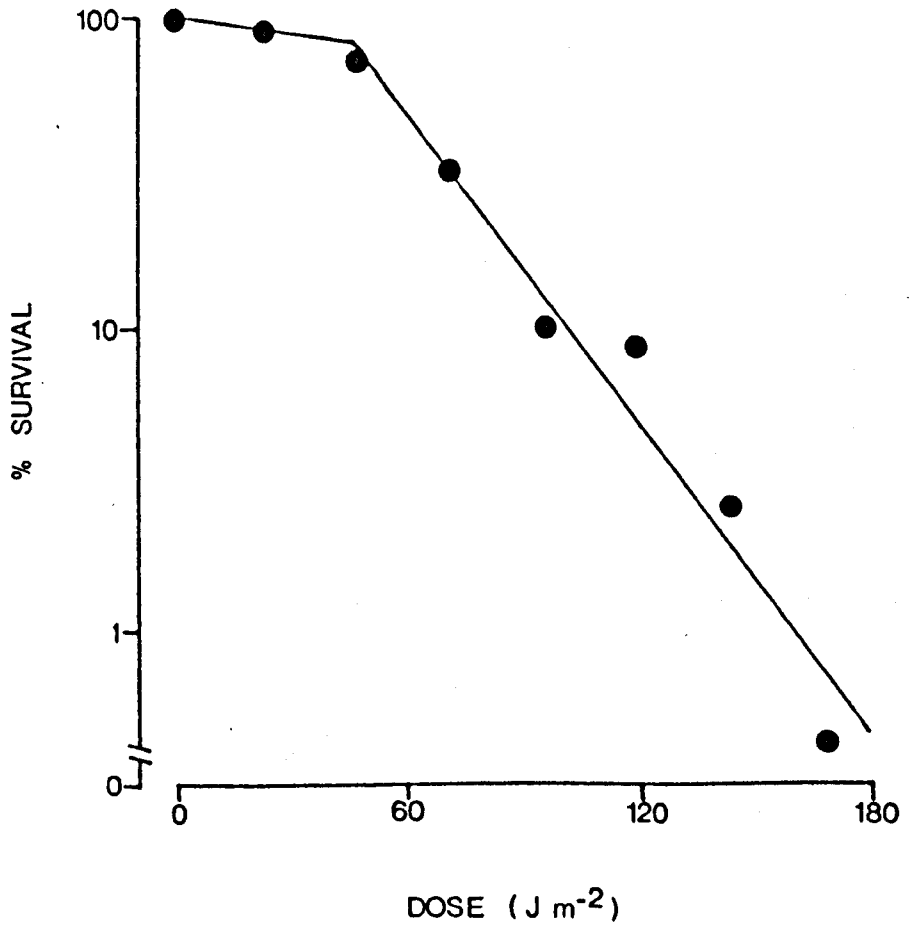


Table 4.1 Germ-tube production by conidia of isolate 22-20, after exposure to UV-light. Survival and numbers of germ-tubes determined after 2 to 6 days incubation at 19°C.

UV DOSE ($J\ m^{-2}$)	NUMBER OF GERMINATED SPORES	NUMBER OF NON-GERMINATED SPORES	GERMINATION (%)	MEAN NUMBER OF GERM-TUBES	SPORES / CLASS No. OF GERM-TUBES 1	2	3	4	5
0	131	10	93.0	2.6	12	53	45	16	5
24	126	21	85.8	2.1	25	70	25	4	2
48	105	55	65.8	1.8	41	5	12	0	1
72	49	115	29.9	1.6	24	21	4	0	0
96	15	151	9.1	1.3	10	5	0	0	0
120	20	231	8.0	1.5	12	6	2	0	0
144	4	163	2.4	1.3	3	1	0	0	0
168	1	239	0.4	-	1	0	0	0	0

Table 4.2 Frequency of mutation to benzimidazole resistance in conidia of isolate 22-20 following UV-irradiation (20 ml spore suspension containing 10^6 spores ml^{-1} in a 90 mm diameter Petri dish).

EXPERIMENT	FUNGICIDE SELECTION		IRRADIATION DOSE ($J\ m^{-2}$) 0	24	48	72	96	120	144
1		Survival (%)*	100	92	71	32	10	9	2
	5μM MBC	Number of mutants	0	0	0	0	4	0	0
		Mutation frequency ($\times 10^{-6}$)**	0	0	0	0	50.0	0	0
2		Survival (%)*	100	100	77	63	24	8	2
	10μM MBC	Number of mutants	0	0	1	2	2	2	0
		Mutation frequency ($\times 10^{-6}$)**	0	0	0.5	1.2	3.1	10.0	0
	20μM TBZ	Number of mutants	0	1	4	4	2	2	0
		Mutation frequency ($\times 10^{-6}$)**	0	0	0.5	2.3	6.3	10.0	0

* Survival after UV-irradiation calculated as a percentage of the unirradiated control

** Mutation frequency calculated as frequency of mutants per surviving conidium.

produced with 96 and 120 J m^{-2} UV-irradiation. At these doses observation of the numbers of germ-tubes produced by individual conidia suggested that the majority of germinating spores had only single viable cells. Consequently a UV dose of 96 J m^{-2} was used in all subsequent experiments for the isolation of auxotrophic mutants.

Maintaining irradiated spores in conditions unsuitable for growth for a period before plating onto the recovery medium to obtain colonies (liquid holding) has been reported to increase the frequency of isolation of mutant types (Rowlands, 1984). The effectiveness of this technique for increasing the number of resistance mutants in P. herpotrichoides was assessed (Table 4.3). Although more resistant mutants were obtained from isolate 22-20 when irradiated spores were kept in sterile distilled water for 24 h, before plating on fungicide-containing medium, the mutation frequency was not increased. Longer periods of liquid holding reduced the number of mutants obtained. Similarly mutant recovery from spores of isolate 22-184 was not greatly enhanced by this procedure.

4.3.2 Isolation of Auxotrophic, Morphological and Pigmentation Mutants

The experimental conditions under which auxotrophic mutations were induced are given for each fungal strain in Appendix IV. On average spores produced on TWA had more cell compartments, and so a greater number of nuclei, than those produced by microcycle conidiation on MYG agar (Table 4.4). These

Table 4.3 Effect of liquid holding on the frequency of mutation to thiabendazole resistance in conidia of 22-20 and 22-184.

ISOLATE	UV DOSE ($J\ m^{-2}$)	PERIOD OF LIQUID HOLDING (h)	FUNGICIDE SELECTION	SURVIVAL (%)	NUMBER OF MUTANTS	MUTATION FREQUENCY ($x10^{-6}$)
22-20	0	0	20 µM TBZ	100	0	<0.1
	96	0	20 µM TBZ	7.4	3	2.1
	96	24	20 µM TBZ	45.1	13	1.4
	96	48	20 µM TBZ	20.6	3	0.7
	96	72	20 µM TBZ	29.0	1	0.2
22-184	96	0	5 µM TBZ	42.4	76	9.4
	96	24	5 µM TBZ	17.4	30	11.0

Table 4.4 Nuclear content of conidia of P. herpotrichoides isolate 22-20, produced on TWA or by microcyclic conidiation on MYG. Spores fixed with 70% ethanol and stained with DAPI overnight at 4°C.

AGAR MEDIUM	NUMBER OF NUCLEI:	PERCENTAGE OF SPORES IN EACH GROUP												
		1	2	3	4	5	6	7	8	9	10	11	12	13
TWA		4	6.5	12	13	17	27	12	4	3	0.5	0.5	0	0.5
MYG	Expt.1	7.5	24	15	19	14	12	5	1	2	0.5	0	0	0
	Expt.2	6	13	16	21	22	8	5	4	3	1	0.5	0.5	0

differences obviously affect the precise level of survival obtained afterirradiation and may, in part, explain the variation in survival level for the different strains. Strains carrying more than one genetic marker were produced either by repeated cycles of mutagenesis, or in the case of some resistance markers,

by selection for spontaneous mutation. Auxotrophic mutants were isolated both by mass screening of surviving colonies following UV-mutagenesis and after enrichment by filtration. The frequency of recovery of auxotrophic mutants ranged between 0.1 and 1.3% of colonies screened, depending on the isolate used. Enrichment for auxotrophs by pre-germination of irradiated conidia in liquid MM and filtration to remove prototrophic mycelium was not successful. Filtration through several layers of muslin (Experiment 1) was ineffective as this material permitted too many hyphal fragments and germinated spores to pass through. Consequently the population of colonies screened was little different from the un-enriched population. The use of glass wool as a filtration medium was equally unsuccessful. Only 5% of the total number of conidia were recovered after filtration, of which 11% were viable compared with 34% of unfiltered spores. Obviously, not only were both germinated and ungerminated conidia removed from the suspension, but many prototrophic spores failed to germinate in the liquid medium.

Liquid holding of spores for 24 h at 19°C. also failed to increase the frequency with which auxotrophic mutants were isolated. Indeed the increased viability of spores after this treatment resulted in decreased rates of mutant isolation. Addition of specific supplements to the recovery medium did not lead to an increased number of auxotrophic mutants being identified, but did result in different classes of auxotrophic requirement in the mutants isolated. Mutants with nutritional requirements for leucine, serine and nicotinic acid were obtained in this way.

Pigmentation and morphological mutants were readily identified amongst the colonies appearing on the recovery plates. Most common among mutants with altered colony colouration on MYG were those with red, yellow or white mycelium. A class of morphological mutants which was repeatedly isolated, both as spontaneous and UV-induced mutants, had spiral hyphal growth, resulting in colonies with curly mycelium (spi). On exposure to light these spiral mutants gradually aquired a deep red pigmentation (Fig. 4.2). All of these colour mutants had normal, hyaline conidia.

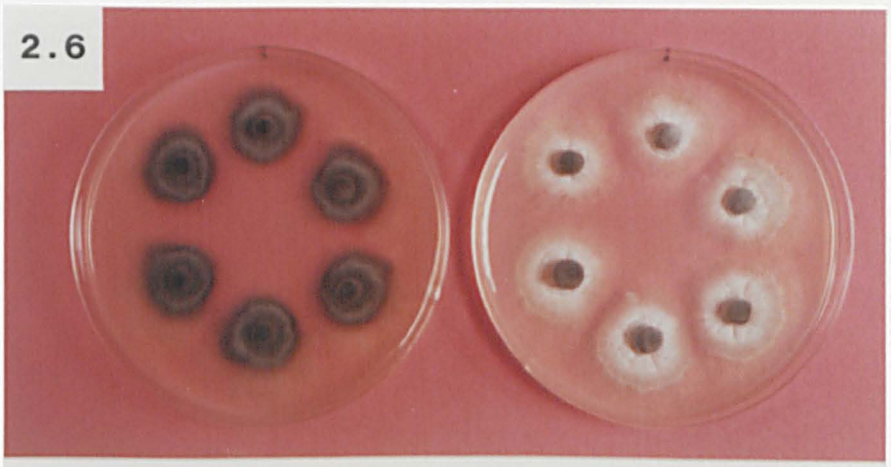
Another class of mutants was regularly obtained in which initial growth on MYG consisted exclusively of microcyclic spore formation (con), producing mycelium only after several days. The conidia produced by these mutants, while appearing morphologically normal, became pigmented at the same time as the mycelium (Fig. 4.3). On MM they appeared unable to form hyphae and remained as small sporulating colonies. Addition of amino acids, either individually or as casamino acids, or supplementation with any of the vitamins or bases failed to promote normal growth. Only when yeast extract was added to the MM was hyphal growth restored. These con mutants were recovered from many isolates of P. herpotrichoides, from both pathotypes, often at high frequency (>10% surviving conidia in one experiment with UV-irradiated spores of isolate 22-12).

The genealogies of mutants derived from isolates 22-20 and 22-12 are presented in Figures 4.4 and 4.5 respectively.

Figure 2.6 Colonies derived from single spores from the BWR-type isolate 22-15 showing variation in pigmentation on MYG.

Figure 4.2 spi mutants obtained from isolate 22-20. When exposed to daylight these colonies aquire a deep red pigmentation.

Figure 4.3 con mutant (22-184) derived from isolate 22-12 growing on MYG. The centre of this colony consists entirely of microcyclically produced conidia, which eventually pigment dark brown. On minimal medium these mutants fail to develop normal mycelium. Bar represents 5 mm.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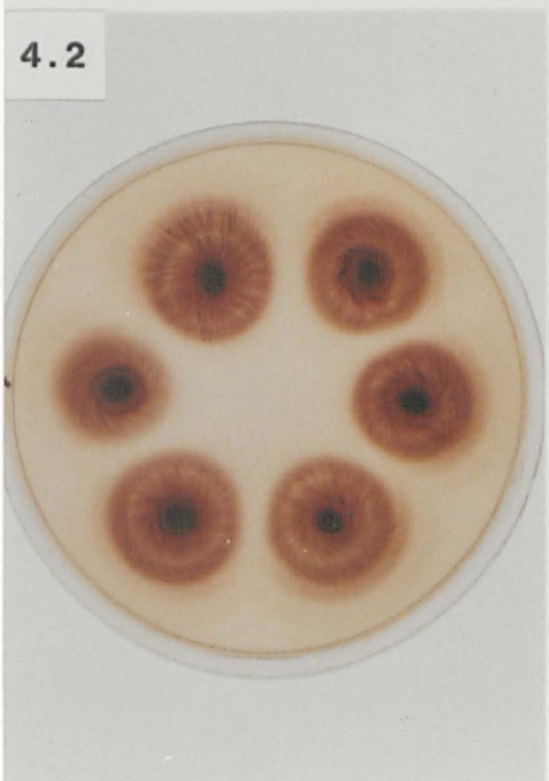

4.2


4.3

FIGURE 4.4. Genealogy of mutants derived from the BW-type isolate 22-20 (spontaneous mutations denoted by *).

GENE SYMBOLS :

ade	adenine requiring
arg	arginine requiring
asn	asparagine requiring
aux	unidentified auxotrophic requirement
ben	benzimidazole resistant
con	microcycle sporulation
fwn	fawn pigmentation
his	histidine requiring
ilv	isoleucine + valine requiring
leu	leucine requiring
lys	lysine requiring
met	methionine requiring
nic	nicotinic acid requiring
orn	ornithine requiring
pnk	pink pigmentation
red	red pigmentation
s	reduced sulphur requiring
ser	serine requiring
spi	spiral growth habit
trp	tryptophan requiring
tyr	tyrosine requiring
ura	uracil requiring
whi	white pigmentation
yel	yellow pigmentation

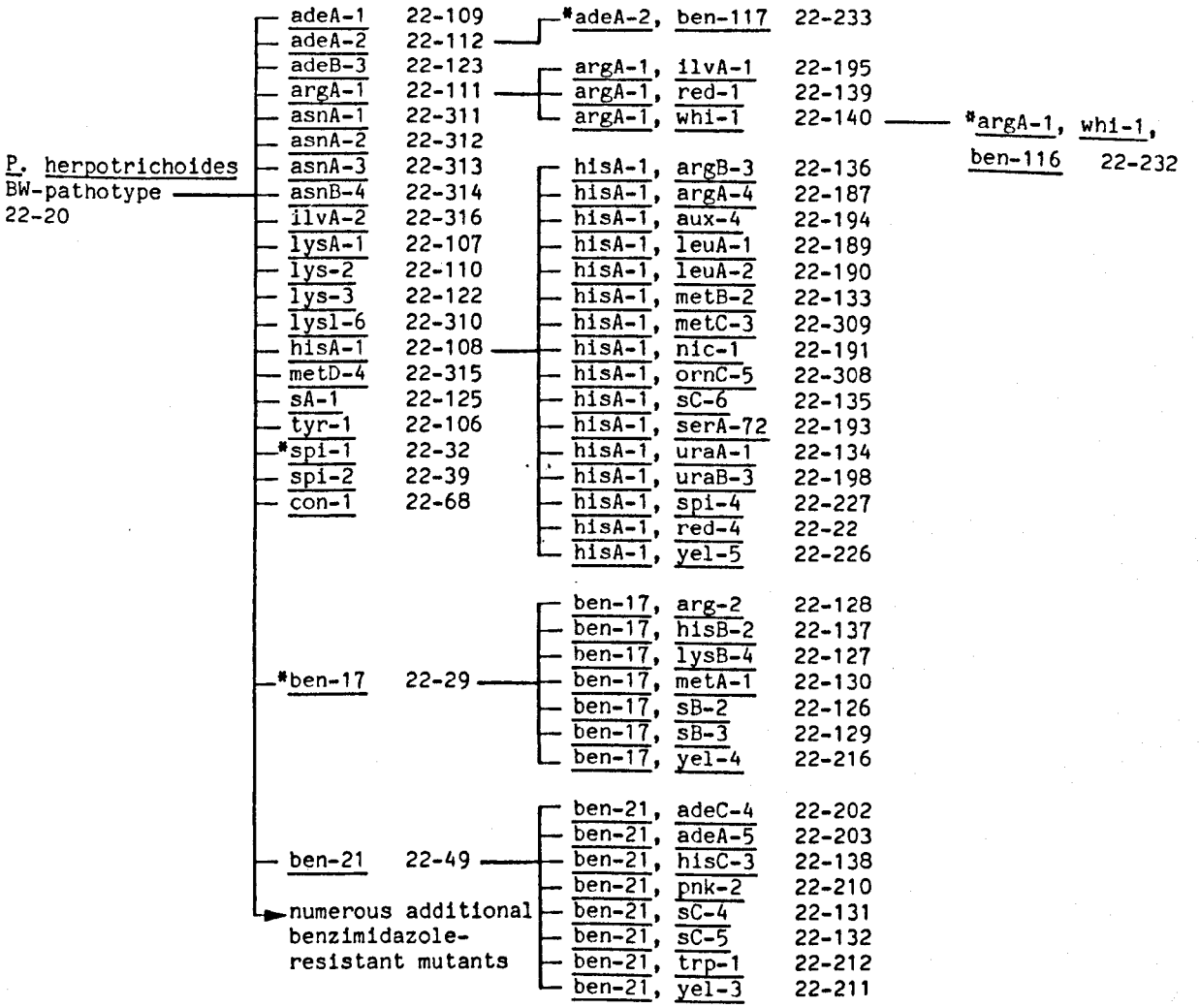
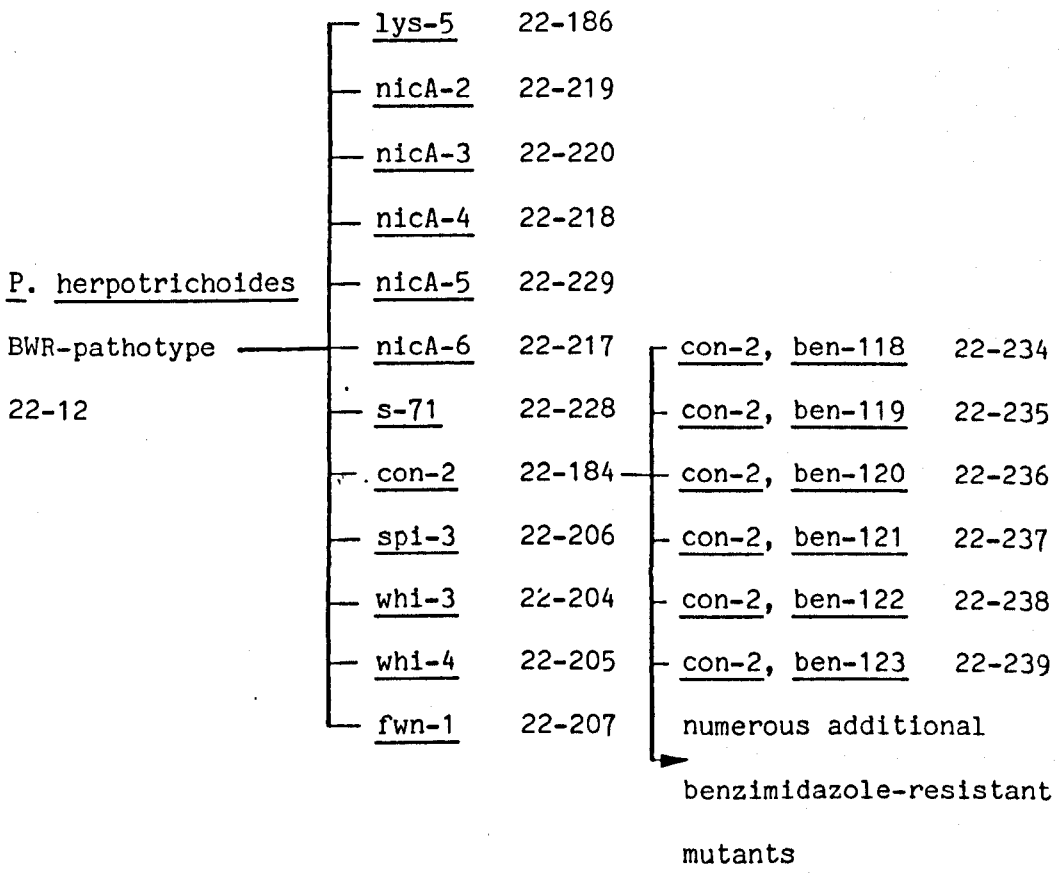

P. herpotrichoides
BW-pathotype
22-20
adeA-1 22-109
adeA-2 22-112
adeB-3 22-123
argA-1 22-111
asnA-1 22-311
asnA-2 22-312
asnA-3 22-313
asnB-4 22-314
ilvA-2 22-316
lysA-1 22-107
lys-2 22-110
lys-3 22-122
lysl-6 22-310
hisA-1 22-108
metD-4 22-315
sA-1 22-125
tyr-1 22-106
*spi-1 22-32
spi-2 22-39
con-1 22-68
*ben-17 22-29
ben-21 22-49
numerous additional benzimidazole-resistant mutants
*adeA-2, ben-117 22-233
argA-1, ilvA-1 22-195
argA-1, red-1 22-139
argA-1, whi-1 22-140
*argA-1, whi-1, ben-116 22-232
hisA-1, argB-3 22-136
hisA-1, argA-4 22-187
hisA-1, aux-4 22-194
hisA-1, leuA-1 22-189
hisA-1, leuA-2 22-190
hisA-1, metB-2 22-133
hisA-1, metC-3 22-309
hisA-1, nic-1 22-191
hisA-1, ornC-5 22-308
hisA-1, sC-6 22-135
hisA-1, serA-72 22-193
hisA-1, uraA-1 22-134
hisA-1, uraB-3 22-198
hisA-1, spi-4 22-227
hisA-1, red-4 22-22
hisA-1, yel-5 22-226
ben-17, arg-2 22-128
ben-17, hisB-2 22-137
ben-17, lysB-4 22-127
ben-17, metA-1 22-130
ben-17, sB-2 22-126
ben-17, sB-3 22-129
ben-17, yel-4 22-216
ben-21, adeC-4 22-202
ben-21, adeA-5 22-203
ben-21, hisC-3 22-138
ben-21, pnk-2 22-210
ben-21, sC-4 22-131
ben-21, sC-5 22-132
ben-21, trp-1 22-212
ben-21, yel-3 22-211

FIGURE 4.5. Genealogy of mutants derived from the BWR-type isolate 22-12 (see Fig. 4.4 for gene symbols)



4.3.3 Characterisation of Auxotrophic Mutants

The auxotrophic mutants identified belonged to eleven main nutritional categories with requirements for: adenine, arginine, asparagine, histidine, isoleucine + valine, leucine, lysine, methionine, nicotinic acid, aromatic amino acids and uracil. The ability to differentiate between non-allelic mutants with the same requirement would increase the number of markers that could be used in genetic analyses. Consequently mutants of the same type were tested for their ability to complement each other, and the characteristics of each mutant assessed by growth on MM containing alternative supplements. Since no biochemical studies of metabolism in P. herpotrichoides have been done, conclusions about the nature of the enzymatic deficiences in these mutants were based on the assumption that the biosynthetic processes in P. herpotrichoides are comparable with those of A. nidulans and N. crassa (Smith & Pateman, 1977; Fincham et al, 1979; Lehninger, 1978).

4.3.3.1 Adenine-requiring Mutants

Five adenine-requiring auxotrophic mutants were obtained in isolate 22-20. All were slightly leaky for growth on MM in the absence of adenine, and four of the mutants were able to utilise hypoxanthine instead of adenine. Complementation studies revealed three complementation groups (Table 4.5).

Table 4.5 Complementation and repair of adenine-requiring auxotrophic mutants.

MUTATION	COMPLEMENTATION GROUP*	MUTANT NUMBER	GROWTH ON MM PLUS**: ADENINE	HYPOXANTHINE
ade-1	A	22-109	+	+
ade-2	A	22-112	+	+
ade-5	A	22-203	+	+
ade-3	B	22-123	+	-
ade-4	C	22-202	+	+

* Mutants in different groups complement each other when co-inoculated on MM, while mutants in the same group did not.
** - = no repair of growth; + = normal growth

The ability of the mutants carrying adeA-1, adeA-2, adeA-5 and adeC-4 to grow in the presence hypoxanthine indicates that the biochemical deficiencies causing auxotrophy in these mutants, occur in the pathway leading to the formation of inosinic acid (IMP). On this basis mutant 22-123 (adeB-3) is likely to be blocked in the conversion of IMP to adenylic acid (AMP; Fig 4.6).

4.3.3.2 Arginine- and Ornithine-requiring Mutants

Five arginine-requiring mutants were isolated from 22-20. One, arg-2 (22-128), was very leaky for growth on MM and was subsequently discarded. The remainder were classified on the basis of mutual complementation into three groups (Table 4.6).

Figure 4.6 Biochemical pathway for purine metabolism. Putative positions of metabolic blocks caused by specific auxotrophic mutations in P. herpotrichoides indicated where known.

Enzymes:

1 adenylosuccinate synthetase

2 adenylosuccinate lyase

3 AMP deaminase

4 nucleosidemonophosphate kinase

5 nucleosidediphosphase kinase

6 adenine phosphoribosyl transferase

7 ribonucleoside diphosphate reductase

8 adenosine kinase

9 adenosine deaminase

10 adenine deaminase

11 hydrolase

12 5'-nucleotidase

13 IMP dehydrogenase

14 GMP synthetase

15 purine nucleoside phosphorylase

16 hypoxanthine phosphoribosyl transferase

17 guanine deaminase

18 xanthine dehydrogenase

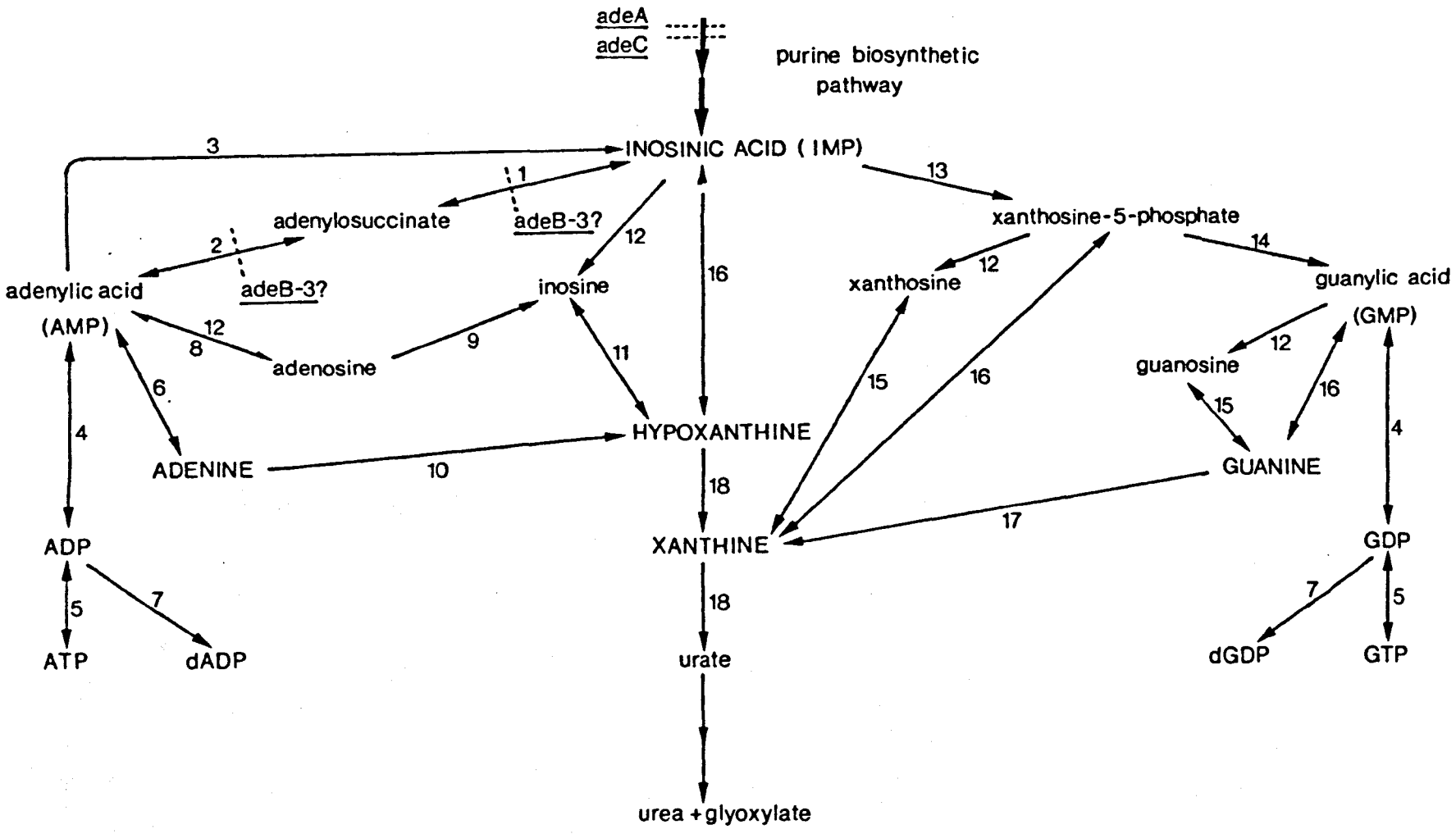

adeA
adeC
purine biosynthetic pathway
INOSINIC ACID (IMP)
adenylosuccinate
adeB-3?
adenylic acid (AMP)
adeB-3?
inosine
adenosine
xanthosine-5-phosphate
xanthosine
guanylic acid (GMP)
guanosine
HYPOXANTHINE
ADENINE
GUANINE
XANTHINE
ADP
ATP
dADP
urate
GDP
dGDP
GTP
urea + glyoxylate
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Table 4.6 Complementation of arginine or ornithine-requiring auxotrophic mutants.

MUTATION	COMPLEMENTATION GROUP	MUTANT NUMBER	GROWTH ON MM PLUS*: N-AGlu	N-AOrn	ORN	CIT	ASucc	ARG	PRO
arg-1	A	22-111	-	-	-	-	-	+	
arg-4	A	22-187	-	-	-	-	-	+	-
arg-3	B	22-136	-	-	-	+	-	+	-
orn-5	C	22-308	-	-	+	+	-	+	(+)

* N-AGlu = N-acetylglutamate; N-AOrn = N-acetlyornithine; ORN = ornithine; CIT = citrulline; ASucc = argininosuccinate; ARG = arginine; PRO = proline; - = no repair of growth; (+) = slightly improved growth; + = normal growth.

Since none of the mutants were able to grow on argininosuccinate, the intermediate between citrulline and arginine, presumably due to poor uptake (Fincham et al, 1979), it is not possible to say which of these biosynthetic steps is likely to be blocked in strains with the argA-1 or argA-4 mutations. Similarly the inability of strain 22-308 to grow on either of the ornithine precursors does not allow a more precise location of the ornC-5 mutation. The argB-3 mutation did not result in a loss of OTC-ase activity, consequently this mutation must block the formation of carbamoyl phosphate, required for the conversion of ornithine to citrulline (Fig 4.7).

4.3.3.3 Asparagine- and Histidine-requiring Mutants

Three histidine- and four asparagine- requiring mutants were identified. All three histidine mutants were complementary. The

Figure 4.7 Metabolic pathway for arginine and proline biosynthesis. Putative positions of metabolic blocks caused by specific auxotrophic mutations in *P. herpotrichoides* indicated where known.

Enzymes:

1 acetylornithine glutamate transacylase

2 acetylglutamate kinase

3 N-acetyl-}'-glutamyl phosphate reductase

4 acetylornithine transaminase

5 ornithine carbamoyl transferase

6 carbamoyl phosphate synthetase (arginine specific)

7 argininosuccinate synthetase

8 argininosuccinate lyase

9 arginase

10 ornithine transaminase

11 glutamic-}'-semialdehyde dehydrogenase

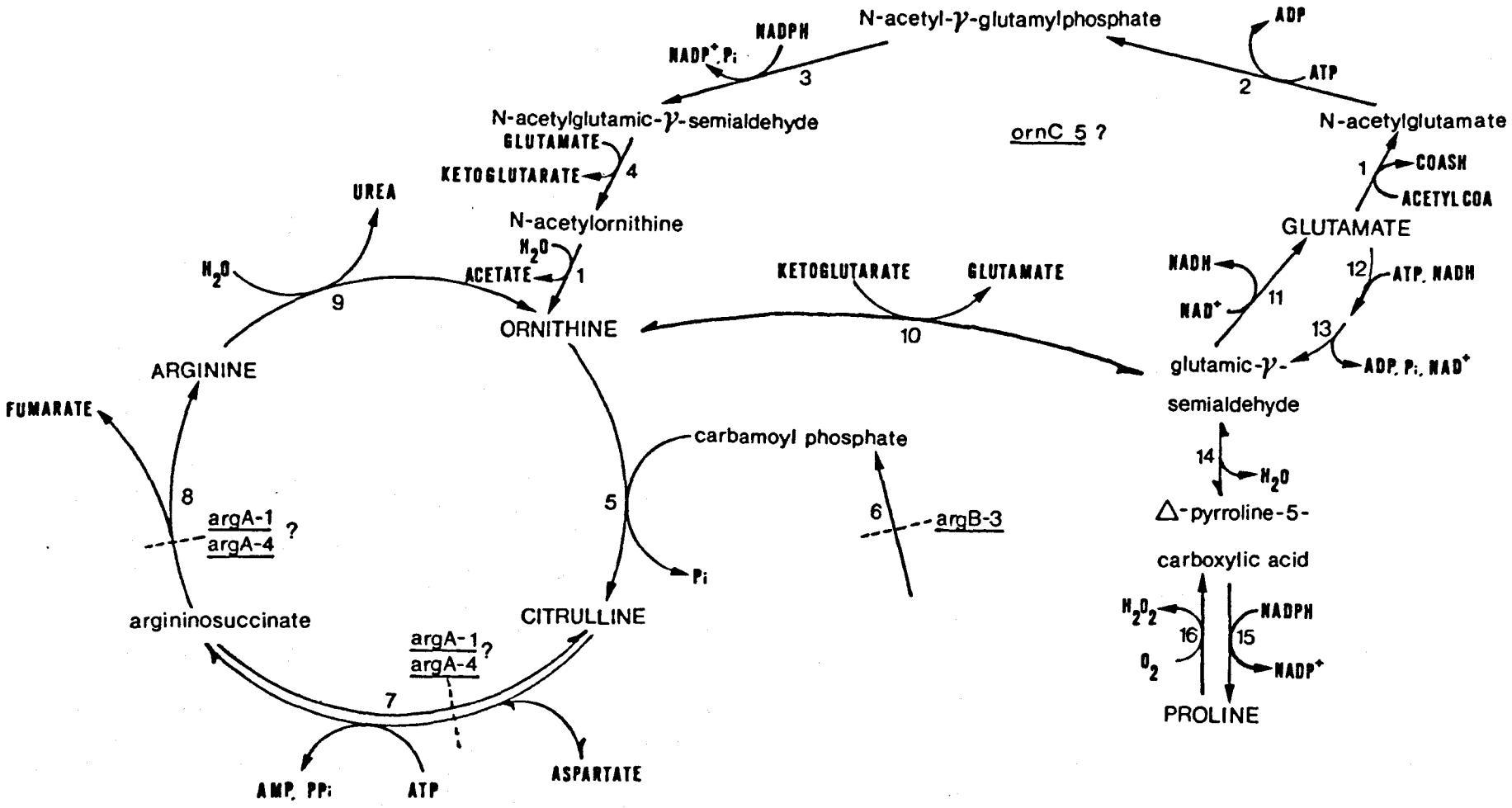
12 glutamate kinase

13 glutamate dehydrogenase

14 spontaneous reaction

15 pyrroline-5-carboxylate reductase

16 proline oxidase


N-acetyl-γ-glutamylphosphate
NADPH
NADP+, Pi
3
ADP
ATP
2
N-acetylglutamic-γ-semialdehyde
ornC 5 ?
N-acetylglutamate
GLUTAMATE
KETOGLUTARATE
4
1
COASH
ACETYL COA
GLUTAMATE
UREA
N-acetylornithine
H2O
H2O
ACETATE
1
NADH
NAD+
11
12
ATP, NADH
KETOGLUTARATE
GLUTAMATE
9
ORNITHINE
10
13
glutamic-γ-
ADP, Pi, NAD+
semialdehyde
ARGININE
14
H2O
FUMARATE
carbamoyl phosphate
8
5
argA-1
argA-4
?
6
argB-3
Pi
Δ-pyrroline-5-
carboxylic acid
argininosuccinate
CITRULLINE
H2O2
16
15
NADPH
O2
NADP+
argA-1
argA-4
?
7
PROLINE
AMP, PPi
ATP
ASPARTATE

asparagine-requiring mutants formed two complementation groups, and were also complementary to the his⁻ mutants. hisA-1 appeared to result in the inability to perform the final step in histidine biosynthesis, since this mutant was unable to utilise histidinol, the immediate precursor of histidine (Table 4.7). The other his⁻ mutations must block histidine biosynthesis at different steps before the formation of histidinol phosphate (Fig.4.8).

Table 4.7 Complementation and repair of histidine- and asparagine-requiring mutants

AUXOTROPHIC CLASS	MUTATION	COMPLEMENTATION GROUP*	MUTANT NUMBER	GROWTH ON MM PLUS**: HIS	HIS-ol	HIS-P	ASN	ASP
HIS⁻	his-1	A	22-108	+	-	-	-	-
	his-2	B	22-137	+	+	+	-	-
	his-3	C	22-138	+	+	+	-	-
ASN⁻	asn-1	A	22-311	-	+	+	+	-
	asn-2	A	22-312	-	+	+	+	-
	asn-3	A	22-313	-	+	+	+	-
	asn-4	B	22-314	+	-	-	+	-

* Grouping based on complementation between mutants in the same class

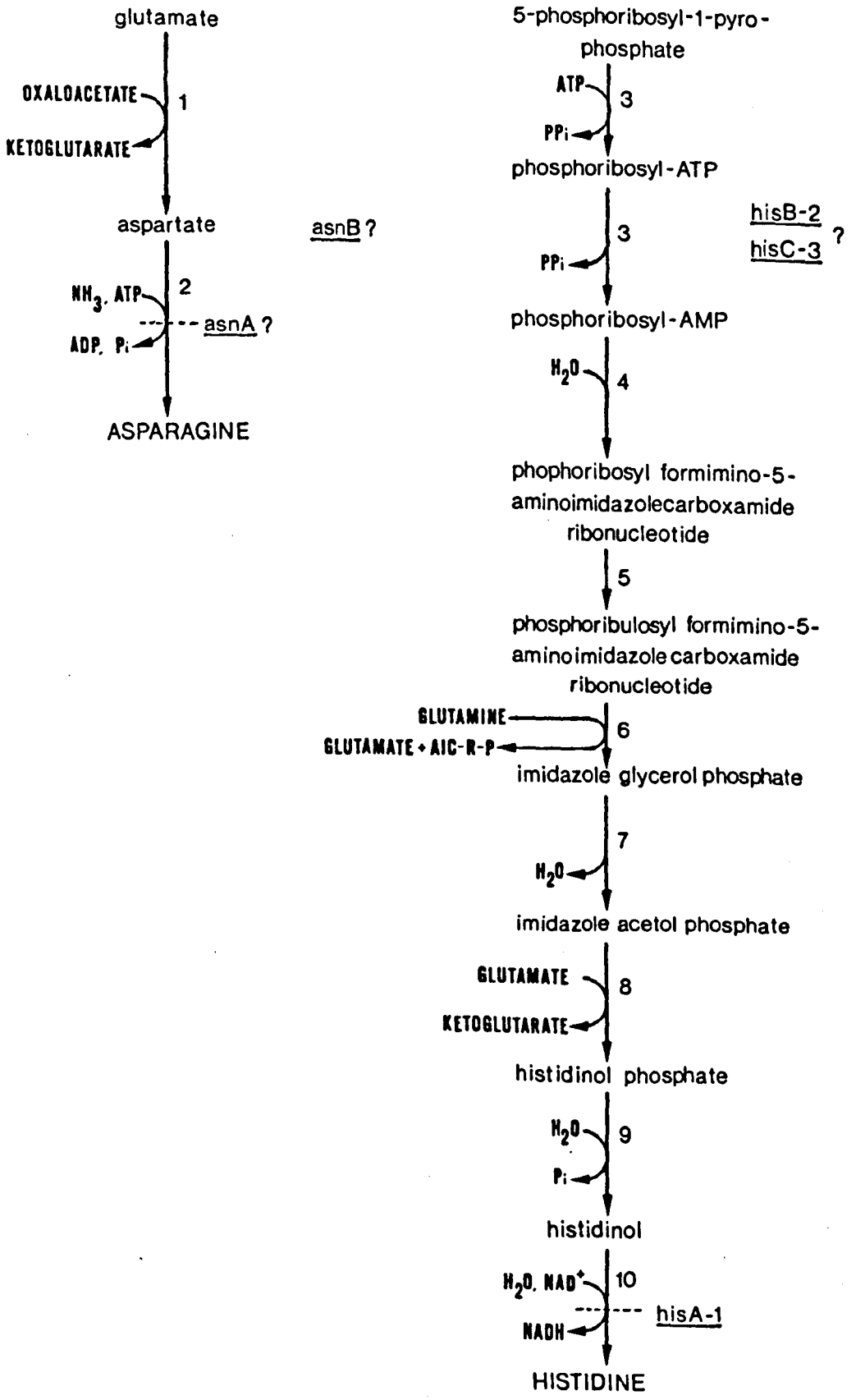
** HIS = histidine; HIS-ol = histidinol; HIS-P = histidinol phosphate; ASN = asparagine; ASP = aspartate; - = no repair of growth;
+ = normal growth.

The pattern of growth repair in the asparagine-requiring mutants was difficult to interpret. asnA-1, asnA-2 and asnA-3, were able to grow on MM in the absence of asparagine when supplied with either of the two histidine precursors, but not when given histidine itself. asnB-4, on the other hand, was able to grow on histidine or asparagine but not when provided with

Figure 4.8 Metabolic pathways for asparagine and histidine biosynthesis. Putative positions of metabolic blocks caused by specific auxotrophic mutations in P. herpotrichoides indicated where known.

Enzymes:

1 glutamate-oxaloacetate aminotransferase
2 asparagine synthetase
3 phosphoribosyl-ATP pyrophosphorylase
4 phosphoribosyl-AMP cyclohydrolase
5 phosphoribosyl formimino-5-aminoimidazole
6 glutamine amidotransferase
7 imidazoleglycerol phosphate dehydratase
8 histidinol phosphate transaminase
9 histidinol phosphate
10 histidinol dehydrogenase


glutamate
OXALOACETATE
1
KETOGLUTARATE
aspartate
asnB ?
NH3, ATP
2
asnA ?
ADP, Pi
ASPARAGINE
5-phosphoribosyl-1-pyro-phosphate
ATP
3
PPi
phosphoribosyl-ATP
3
hisB-2
hisC-3
?
PPi
phosphoribosyl-AMP
H2O
4
phophoribosyl formimino-5-aminoimidazolecarboxamide ribonucleotide
5
phosphoribulosyl formimino-5-aminoimidazole carboxamide ribonucleotide
GLUTAMINE
6
GLUTAMATE + AIC-R-P
imidazole glycerol phosphate
7
H2O
imidazole acetol phosphate
GLUTAMATE
8
KETOGLUTARATE
histidinol phosphate
H2O
9
Pi
histidinol
H2O, NAD+
10
hisA-1
NADH
HISTIDINE

histidinol or histidinol phosphate. The reasons for these responses remains unclear. The possibility that the asaragine was contaminated with histidine, so allowing growth of the asnB-4 mutant, which would therefore be a histidine-requiring mutant, was not supported by the growth responses of the three his$^-$ mutants, none of which produced any growth on asparagine. Conversely the histidinol and histidinol phosphate may have contained asparagine, enabling the other asn$^-$ mutants to grow. However, in this case, strain 22-314 (asnB-4) should have been able to grow on these supplements. An alternative theory, that 22-314 was a mixed culture, did not appear to be the case either. No heterokaryotic growth was produced by this strain and repeated purification by single spore isolation failed to reveal differing phenotypes.

4.3.3.4 Lysine-requiring Mutants

Six lysine-requiring mutants were produced, five from isolate 22-20, one of which (lys-2) was too leaky on MM to be useful, and one from isolate 22-12. Three mutants from 22-20 were found to be complementary, the fourth could not be assigned to any complementation group (Table 4.8). Similarly the mutant derived from 22-12 (lys-5) did not complement any of the other mutants, this will be discussed at greater length in Chapter 7. None of the lysine-requiring mutants was able to utilise α-aminoadipate in place of lysine, though whether this was due to impaired uptake or the location of the biochemical blocks was not clear.

4.3.3.5 Methionine-, Reduced Sulphur- and Serine-requiring Mutants

The most frequently recovered class of auxotrophs consisted of mutants deficient in sulphur metabolism. These could be classified as methionine-requiring or as reduced sulphur-requiring, depending on their growth on various sulphur sources (Table 4.9). Additionally, one mutant (22-193) appeared to be deficient for serine biosynthesis. This mutant was able to grow normally when provided with serine, cysteine or methionine, and was leaky on MM.

Table 4.8 Complementation and repair of lysine-requiring mutants.

PARENTAL ISOLATE	MUTATION	COMPLEMENTATION GROUP	MUTANT NUMBER	GROWTH ON MM PLUS*: LYS	AAA
22-20	lys-1	A	22-107	+	-
	lys-4	B	22-127	+	-
	lys-6	C	22-310	+	-
	lys-3	?	22-122	+	-
22-12	lys-5	?	22-186	+	-

* LYS = lysine; AAA = alpha-aminoadipate; - = no repair of growth; + = normal growth.

All four methionine-requiring mutants were cross compatible. On the basis of growth on the various sulphur-containing amino acids and their intermediates, the metA-1 mutation appears to block the conversion of cystathione into homocysteine (Fig. 4.9). metB-2 may cause a block in the last step in methionine synthesis, while metC-3 seems to prevent the conversion of cystathione into homocysteine. The basis of the

Figure 4.9 Metabolic pathway for cysteine and methionine biosynthesis. Putative positions of metabolic blocks caused by specific auxotrophic mutations in P. herpotrichoides indicated where known.

Enzymes:

1 sulphate permease

2 sulphate adenyltransferase

3 adenyl sulphate kinase

4 PAPS reductase

5 sulphite reductase

6 acetylserine sulphydrilase

7 cystathionine-γ-synthetase

8 cystathionine-β-lyase

9 homocysteine methyltransferase

10 methionine adenosyltransferase

11 methyltransferase

12 adenosyl homocysteinease

13 cystathionine-β-synthetase

14 cystathionine-γ-lyase

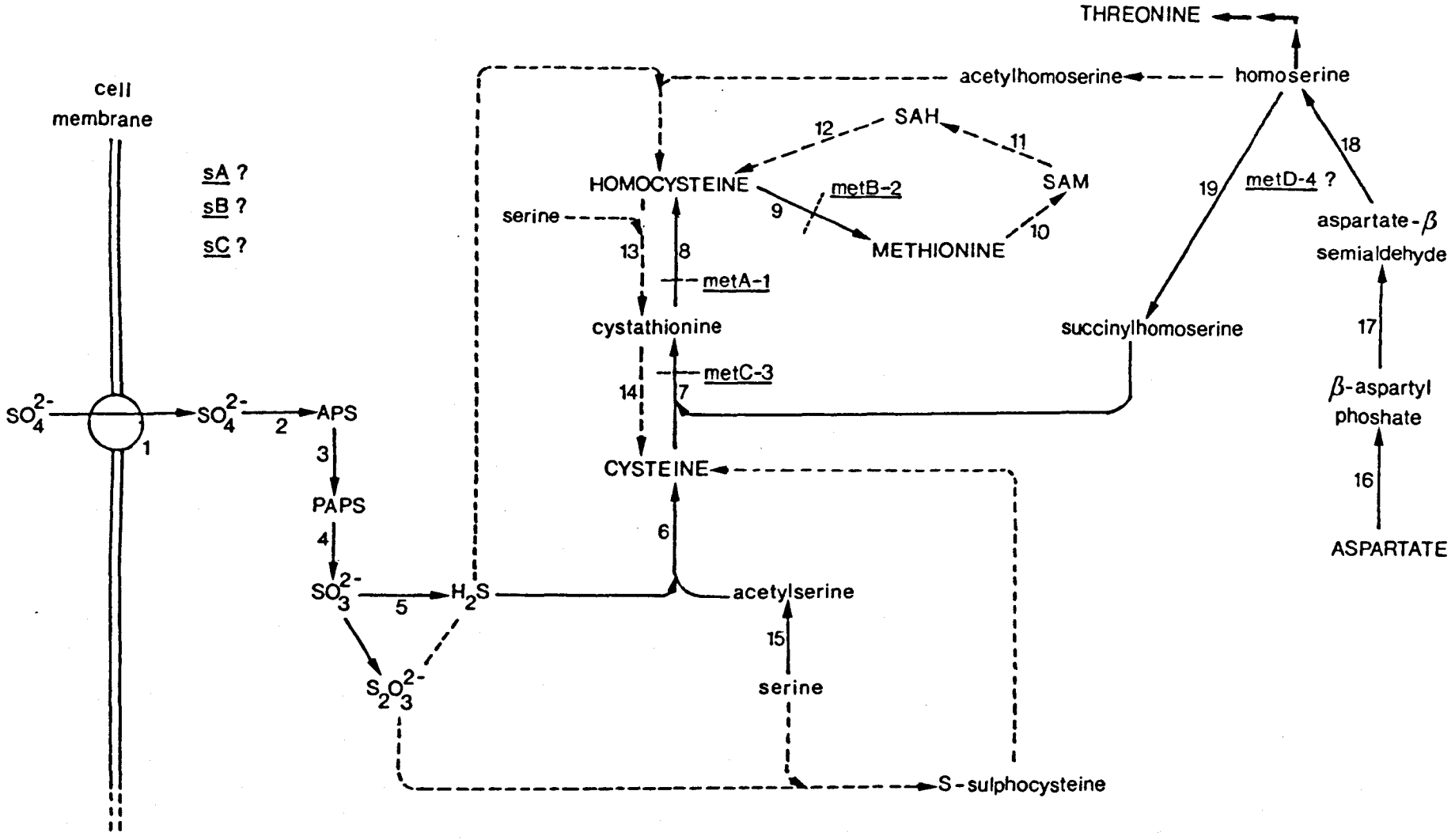
15 serine acetyltransferase

16 aspartate kinase

17 aspartate-β-semialdehyde dehydrogenase

18 homoserine dehydrogenase

19 homoserine acyltransferase


THREONINE
homoserine
acetylhomoserine
cell membrane
sA ?
sB ?
sC ?
SAH
12
11
18
HOMOCYSTEINE
metB-2
SAM
19
metD-4 ?
aspartate-β semialdehyde
serine
9
10
13
8
METHIONINE
metA-1
cystathionine
succinylhomoserine
17
metC-3
14
7
β-aspartyl phoshate
SO_4^{2-}
SO_4^{2-}
APS
2
1
3
CYSTEINE
16
PAPS
6
4
ASPARTATE
SO_3^{2-}
H_2S
5
acetylserine
15
$S_2O_3^{2-}$
serine
S-sulphocysteine

methionine auxotrophy caused by metD-4 is less obvious, but may involve altered synthesis of homoserine, since supplementation with this compound did allow some growth on MM. Three complementation groups were recognised amongst the reduced sulphur-requiring mutants, but little can be said about the basis of the auxotrophy in these strains. All produced leaky growth on

Table 4.9 Complementation and repair of methionine- and reduced sulphur-requiring mutants.

MUTATION	COMPLEMENTATION GROUP*	MUTANT NUMBER	GROWTH ON MM PLUS**: SO_3^{--}	$S_2O_3^{--}$	CYS	CYT	HCys	MET	SER	HSer
met-1	A	22-130	-	-	-	-	(+)	+	-	-
met-2	B	22-133	-	-	-	-	-	+	-	-
met-3	C	22-309	-	-	-	+	(+)	+	-	-
met-4	D	22-315	-	-	-	(+)	(+)	+	-	(+)
s-1	A	22-125	-	+	+	NT	+	+	-	-
s-2	B	22-126	-	+	+	NT	(+)	+	-	-
s-3	B	22-129	-	+	+	NT	+	+	-	-
s-4	C	22-131	-	+	+	NT	(+)	+	-	-
s-5	C	22-132	-	+	+	NT	+	+	-	-
s-6	C	22-135	-	+	+	NT	+	+	-	-
***s-71	?	22-228	-	+	+	NT	+	+	-	-
ser-72	A	22-193	-	-	+	NT	(+)	+	+	-

* Grouping based on complementation between mutants of the same class

** SO_3^{--} = sodium sulphite; $S_2O_3^{--}$ = sodium thiosulphate; CYS = cysteine; CYT = cystathione; HCys = homocysteine; MET = methionine; SER = serine; HSer = homoserine; - = no or leaky growth; Slight or moderate growth; + = normal growth.

*** Mutant derived from isolate 22-12.

MM. None, however, resulted in resistance to selenate, an inhibitor of sulphate metabolism (See Chapter 7). Mutant 22-193, carrying serA-72, complemented all the s^- mutants, with the sole exception of s-71. This mutation occurred in a strain derived from isolate 22-12, and did not complement any of the other sulphur mutations.

4.3.3.6 Leucine- and Isoleucine + valine-requiring Mutants

Two leucine and two isoleucine + valine auxotrophs were isolated. No complementation was found between either pair of mutants, although leucine-requiring strains complemented the ilv^- mutants. No repair of auxotrophy was produced when isoleucine and valine were replaced by their immediate precursors (Table 4.10). Either these compounds are not taken up, or the ilv^- mutants are deficient for the enzyme valine transaminase, which catalyses the final step in the biosynthesis of these two amino acids (Fig. 4. 10).

4.3.3.7 Uracil-, Nicotinic acid-, Tyrosine- and Tryptophan-requiring Mutants

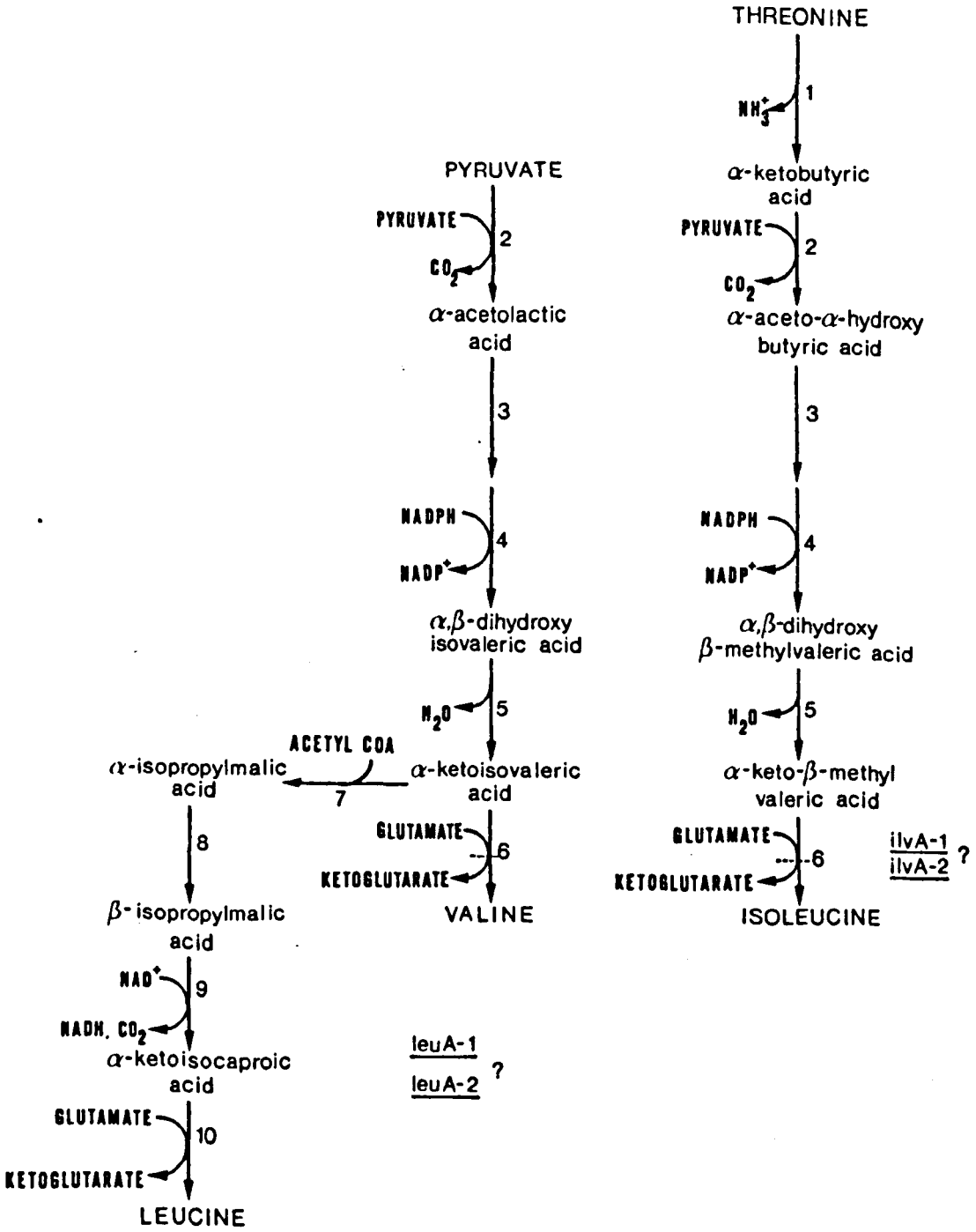
Both pyrimidine-requiring mutants obtained were slightly leaky on MM, and were complementary (Table 4.11). Even more leaky growth was exhibited by the tyrosine- and tryptophan-requiring mutants. On MM lacking the appropriate supplements the growth rates of these two mutants were approximately half that when the supplements were added.

Five nicotinic acid requiring mutants were recovered from isolate 22-12, and one from isolate 22-20. No complementation was

Figure 4.10 Metabolic pathway for valine, isoleucine and leucine biosynthesis. Putative positions of metabolic blocks caused by specific auxotrophic mutations in P. herpotrichoides indicated where known.

Enzymes:

1 threonine dehydratase
2 acetolactate synthase
3 acetolactate mutase
4 reductase
5 dihydroxy acid dehydratase
6 valine transaminase
7 α-isopropylmalate synthase
8 α-isopropylmalate dehydratase
9 isopropylmalate dehydrogenase
10 leucine transaminase


THREONINE
NH3+
1
α-ketobutyric acid
PYRUVATE
CO2
2
α-aceto-α-hydroxy butyric acid
3
NADPH
NADP+
4
α,β-dihydroxy β-methylvaleric acid
H2O
5
α-keto-β-methyl valeric acid
GLUTAMATE
KETOGLUTARATE
6
ilvA-1
ilvA-2
?
ISOLEUCINE
PYRUVATE
CO2
2
α-acetolactic acid
3
NADPH
NADP+
4
α,β-dihydroxy isovaleric acid
H2O
5
ACETYL COA
7
α-isopropylmalic acid
α-ketoisovaleric acid
GLUTAMATE
KETOGLUTARATE
6
VALINE
8
β-isopropylmalic acid
NAD+
9
NADH, CO2
α-ketoisocaproic acid
leuA-1
leuA-2
?
GLUTAMATE
KETOGLUTARATE
10
LEUCINE

seen between any of these six mutants, and none were repaired by tryptophan.

Table 4.10 Complementation and repair of leucine- and isoleucine + isoleucine-requiring mutants.

MUTATION	COMPLEMENTATION GROUP*	MUTANT NUMBER	GROWTH ON MM PLUS**: LEU	ILE	VAL	ILE +VAL	ILE +KIV	VAL +KMV	KIV +KMV
leu-1	A	22-189	+	-	-	-	-	-	-
leu-2	A	22-190	+	-	-	-	-	-	-
ilv-1	A	22-195	-	-	-	+	-	-	-
ilv-2	A	22-316	-	-	-	+	-	-	-

* Grouping based on complementation between mutants of the same class.
** LEU = leucine; ILE = isoleucine; VAL = valine; KIV = α-keto- iso-valeric acid; KMV = α-keto- β-methyl-valeric acid; - = no repair of growth; + = normal growth.

Table 4.11 Complementation and repair of pyrimidine-requiring mutants.

MUTATION	COMPLEMENTATION GROUP	MUTANT NUMBER	GROWTH ON MM PLUS*: URA	URID	CYTO	THY
ura-1	A	22-134	+	+	+	-
ura-3	B	22-198	+	+	+	-

* URA = uracil; URID = uridine; CYTO = cytosine; THY = tyhmine; - = no repair of growth; + = normal growth.

4.4 DISCUSSION

Initial doubts about the use of multicellular spores for the isolation of mutants were unfounded. Mutation frequency, assessed by selection for benzimidazole resistance was greatest at UV doses of 96 to 120 J $m^{-2}s^{-1}$. However, the optimum irradiation dose for the isolation of auxotrophic mutants may not be the same as that for resistance mutants, as the presence of viable prototrophic nuclei in the conidium will preclude the isolation of the auxotroph, whereas the occurrence of fungicide sensitive nuclei in the same mycelium is unlikely to prevent the identification of dominant resistant mutants. Despite this auxotrophic mutants were successfully isolated at frequencies comparable with those recorded for other fungal species (Bertoldi & Caten, 1975; Hastie & Heale, 1984).

The types of auxotrophic mutants isolated were similar to those described for other species (Bertoldi & Caten, 1975; Churchill & Mills, 1984; Hastie & Heale, 1984). The range of requirements obtained may be more the result of the experimental procedure than the mutability of any particular locus, since alteration of the composition of the recovery medium enabled a different spectrum of requirements to be recovered. On the other hand the large number of nicotinic acid-requiring mutants derived from isolate 22-12 may indicate differential mutability of loci in this strain. Puhalla (1980) reported a high frequency of *nic*$^-$ mutants amongst auxotrophs induced in *Verticillium dahliae*.

Microcylic sporulation has been described in several species

(Smith et al, 198la,b), usually in response to some environmental or nutritional factor. In Fusarium solani f.sp. pisi mutants with a stable microcycle were produced by a two-step mutagenic procedure (Kolmark, 1984), the first step blocking the formation of macroconidia, and the second eliminating the need for hyphal development before production of the microconidia. The con mutants of P. herpotrichoides were readily isolated after mutagenic treatment and were occasionally obtained as spontaneous mutants, in many of the field isolates. The high frequency with which they appeared after UV-irradiation of spores suggests a particularly mutable genetic "switch" controlling morphogenesis. In addition, the observations that con mutants are unable to form normal mycelium on MM, and that microcyclic spore formation in wild-type isolates on MYG agar is sustainable for 2 to 3 generations at the most before reversion to the mycelial form, strongly indicate that nutrition plays an important role in the control of microcyclic conidiation in this fungus.

CHAPTER 5

ISOLATION AND REGENERATION OF PROTOPLASTS

5.1 INTRODUCTION

Fusion of fungal protoplasts provides a method whereby gene exchange can be artifically induced. Following the successful application of protoplast techniques to the genetic study and manipulation of industrially important species (Kevei & Peberdy, 1977, 1979; Keller et al, 1980; Peberdy, 1980; Ferenczy, 1981; Ferenczy & Pesti, 1982; Toyama et al, 1984; Yoo et al, 1984, 1985; Birkett & Hamlyn, 1985; Hamlyn et al, 1985; Magae et al, 1985, 1986; Reymond & Fevre, 1986; Reymond, Veau & Fevre, 1986), considerable interest has been shown in the use of these techniques with plant pathogenic fungi (Garcia-Acha et al, 1966; Bartnicki-Garcia & Lippman, 1966; Lopez-Belmonte et al, 1966; de Waard, 1976; Harris, 1982; Hashiba & Yamada, 1982, 1984; Typas, 1983; Levitin et al, 1984; Lynch et al, 1985a, b; Molnar et al, 1985; Morehart et al, 1985; Newton & Caten, 1985; Zhemchuzhina & Dzhavakhiya, 1985; Gwinn & Daub, 1986). In many facultative biotrophic fungi naturally occurring sexual gene exchange is either absent (Hashiba & Yamada, 1982; Typas, 1983) or recalcitrant and therefore not amenable to manipulation in the laboratory (Harris, 1982; Leslie, 1983).

Protoplast fusion also allows the basis of heterokaryon incompatibility to be investigated. Mechanisms of vegetative incompatibility which are based in the cell wall can be overcome by this method, enabling strains to be crossed which would not be possible using orthodox procedures (Dales & Croft, 1977; Typas,

1983; Jackson & Heale, 1983; Hastie & Heale, 1984; Zhemchuzhina et al, 1985). In addition fungal protoplasts are widely used in molecular biology, both as a source of genetic material and for transformation with cloned DNA (Hynes, 1986).

This chapter describes the development of a protoplast isolation and regeneration system for P. herpotrichoides. The main factors affecting protoplast release, including mycelial age, lytic enzyme combination and choice of osmotic stabilizer have all been optimised, and conditions for high frequency protoplast regeneration determined.

5.2 MATERIALS AND METHODS

5.2.1 Strains

Isolate 22-20, a fast-growing BW-type strain, was used throughout for the development of a protoplast culture system. The origin of this isolate is given in Appendix 1.

5.2.2 Production of Mycelium for Protoplast Isolation

For the initial screen of commercial enzymes for lytic activity against P. herpotrichoides, mycelium was produced on sterile cellophane sheets spread over malt extract-glucose agar. Cellophane cultures were grown for 4 days at 19°C from small agar plug inocula. In all subsequent experiments mycelium grown in shake culture was used. The liquid medium (MYG liquid) was composed as follows: (g l^{-1}) malt extract, 5; yeast extract, 2.5; glucose, 10; and adjusted to pH 4.0 prior to sterilisation in 25 ml volumes in 250 ml flasks. Media were autoclaved at 121°C for 30 min before use. At first mycelium was grown from an inoculum

of 2 x 10^4 spores ml^{-1} and cultured for up to 90 h. Once a suitable enzyme combination had been found the inoculum concentration was increased to 10^5 spores ml^{-1}, and cultures maintained for up to 44 h. All flask cultures were incubated on an orbital shaker at 19°C and 100 rpm.

5.2.3 Enzyme Solutions

Unless stated otherwise all enzymes were dissolved in 0.05 M sodium maleate buffer (pH 5.8) containing 0.6 M KCl as the osmotic stabilizer. Stock solutions were prepared with 15 mg enzyme ml^{-1} and diluted with sterile buffered stabilizer where required, or by combination with other enzyme solutions, to give a final concentration of 5 mg ml^{-1} for each enzyme. Enzyme solutions were sterilized by centrifugation at 30000 x g and 4°C for 30 min, and stored frozen at -20°C.

5.2.4 Protoplast Formation

When cellophane-grown mycelium was used single colonies were excised, washed by immersion in stabilizer and placed in the appropriate enzyme solution (0.6 ml volumes in sterile Eppendorf tubes). Mycelium from shake cultures was harvested aseptically by vacuum filtration, washed twice with distilled water and once with osmotic stabiliser. Where a specific quantity of mycelium was required, the mycelial mat was press-dried, weighed and then resuspended at the appropriate concentration in the enzyme solution. Otherwise 1 ml aliquots of mycelial suspension were transferred to centrifuge tubes and the mycelium recovered by centrifugation at 3000 x g for 5 min. The lytic mixtures were

incubated at 28°C with gentle shaking for at least 3 h. Protoplast numbers were determined using a Neubauer counting chamber.

5.2.5 Protoplast Regeneration

Protoplast regeneration was compared on complete and minimal medium using various compounds as osmotic stabilisers. Glucose and osmotic stabilizer were autoclaved separately and added to the medium prior to use. Protoplasts produced after 3 to 24 h digestion in the enzyme solution were recovered by filtration through sinter glass filters (porosity 2), followed by centrifugation at 3000 x g for 5 min. After removal of the enzyme solution protoplasts were suspended in the stabilizer solution and diluted to give 10^4 protoplasts ml^{-1}. One hundred ul portions of the protoplast suspension were spread on complete or minimal medium containing an appropriate concentration of stabilizer. Regenerating protoplasts were incubated at 19°C. Regeneration frequency was expressed as the percentage of protoplasts, of the total microscopic count, that formed colonies after 4-28 days of culture. Control platings of lysed protoplast suspensions were also made.

5.2.6 Enzyme Assays

Enzyme activities were determined under conditions similar to those used for protoplast release; enzymes and substrates were dissolved in 0.5 M sodium maleate buffer, and assays were carried out at 28 °C, except for chitinase which was determined at 37°C. Stabilizer, however, was not included in case its presence interfered with the detection of the products of hydrolysis.

Chitinase activity was determined using acid-swollen chitin (prepared as described by Hamlyn, 1982) as substrate. The liberated 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine) was measured by the method of Reissig et al (1955) using N-acetylglucosamine (Sigma) as standard.

β-D-glucanase activity was determined using laminarin (Koch-Light) as the substrate. α-D-glucanase activity was measured using nigeran (Koch-Light) and mutan (Novo Industries) as the substrate. Bakers' yeast (Sigma) mannan was used as the substrate to determine α-D-mannanase activity. In each case the liberation of reducing sugars was measured by the 3,5-dinitrosalicylic acid method (Miller et al, 1960) using D-glucose as standard.

Protease activity expressed as trypsin equivalents, was determined by the method of de Vries (1974) with Hammarsten casein, denatured by boiling for 15 min, as substrate.

5.2.7 Protein Determination

The protein content of the enzyme preparations was assessed with the Folin-Ciocalteau phenol reagent by the method of Lowry et al (1951). Bovine serum albumen (Sigma) was used as the standard.

5.2.8 Enzymes and Chemicals

The enzymes tested for lytic activity against P. herpotrichoides are listed in Table 5.1 with details of the supplier and, where known, the source organism from which they were obtained.

Laminarin and nigeran were obtained from Koch-Light Laboratories, Colnbrook. Brewers' yeast mannan, chitin, bovine

serum albumin and N-acetylglucosamine were purchased from the Sigma, London Chemical Company Ltd., Poole. Folin-Ciocalteau phenol reagent, 3,5-dinitrosalicylic acid and Hammarsten casein were from BDH Chemicals Ltd., Poole. All other chemicals were of analytical reagent grade and either from BDH Chemicals Ltd., Poole, or Fisons plc, Loughborough.

Table 5.1 Enzymes screened for protoplast production from *P. herpotrichoides*

ENZYME PREPARATION	SOURCE ORGANISM	MANUFACTURER/SUPPLIER*
Cellulase CP	*Penicillium funiculosum*	John & E. Sturge Ltd., Selby, North Yorkshire
Cellulase "onozuka" R-10 Cellulase RS	*Trichoderma viride*	Yakult Honsha co. Ltd, 8-21 Singikancho, Nishinomiya Hyogo T662, Japan.
Macerozyme R-10	*Rhizopus* sp.	*Genetic Research Instrumentation Ltd. Lynton, Parsonage Rd. Takeley, Bishops Stortford, Herts CM22 610
Cellulase (Mayvil)	*Aspergillus niger*	Mayvil Chemicals Ltd., Rookery Bridge, Sandbach, Cheshire CW11 9QZ
Cellulase (Boehringer)	*T. viride*	*BCL. The Boehringer Corporation (London) Ltd. Bell Lane, Lewes, East Sussex BN7 1LG.
Cellulysin	*T. viride*	*Calbiochem-Behring Corporation, C-P Laboratories Ltd., P.O. Box 22, Bishops Stortford, Herts.
Driselase	*Irpex lacteus*	Kyowa Hakko Kiogo Co. Ltd. Ohtemachi Building, Ohtemachi, Chiyoda-Ku, Tokyo, Japan. *Genetic Research Instrumentation. Ltd.

Table 5.1 continued

Driselase	Irpex lacteus	*Fluka, A.G. Fluorochem. Ltd. Park Dale Road, Glossop, Derbyshire. *Sigma Chemical Co. Ltd. Fancy Road, Poole, Dorset, BH17 7NH
β-Glucanase Lytic Enzyme L1	P. emersonii	*BDH Chemicals Ltd. Fourways, Carlyon Ind. Est. Atherstone, Warwicks, CV9 1JG.
Helicase	Helix pomatia	I'Industrie Biologique francaise, Clichy, France.
Meicelase P	T. viride	Meiji Seika Kaisha Ltd., Pharmaceutical Division, 8,2 Chome Kyob[illegible] Chuo-ko. Tokyo, [illegible]an. *Genetic Research Instrumentation Ltd.
Novozym 234	T. harzianum	Novo Industri A/S, Enzyme Division, Bagsvaard, Denmark.
Pectinase	A. niger	*Serva feinbiochemica Uniscience Ltd., Uniscience House, 8 Jesus Lane, Cambridge CB5 8BA
Pectolyase Y23	A. japonicus	Seishin Pharmaceutical Co. Ltd., 4-13 Koami-cho, Nichonbashi, Chwo,ko, Tokyo, Japan. *Genetic Research Instrumentation Ltd.
Rhozyme HP150	Unknown	Rohm & Haas co., Independence Mall West, Philadelphia, PA 19105, U.S.A. *Pollock & Poole Ltd. Ladbroke Close, Woodley, Reading, RG5 4DX.
Rolament P	Unknown	Rohm GmbH Chemishe Fabrik, Postfach 4242, 6100 Darmstadt, Germany.
Snail Enzyme	Helix pomatia	Dept. of Microbiology, University of Szeged, Szeged, Hungary.

5.3 RESULTS

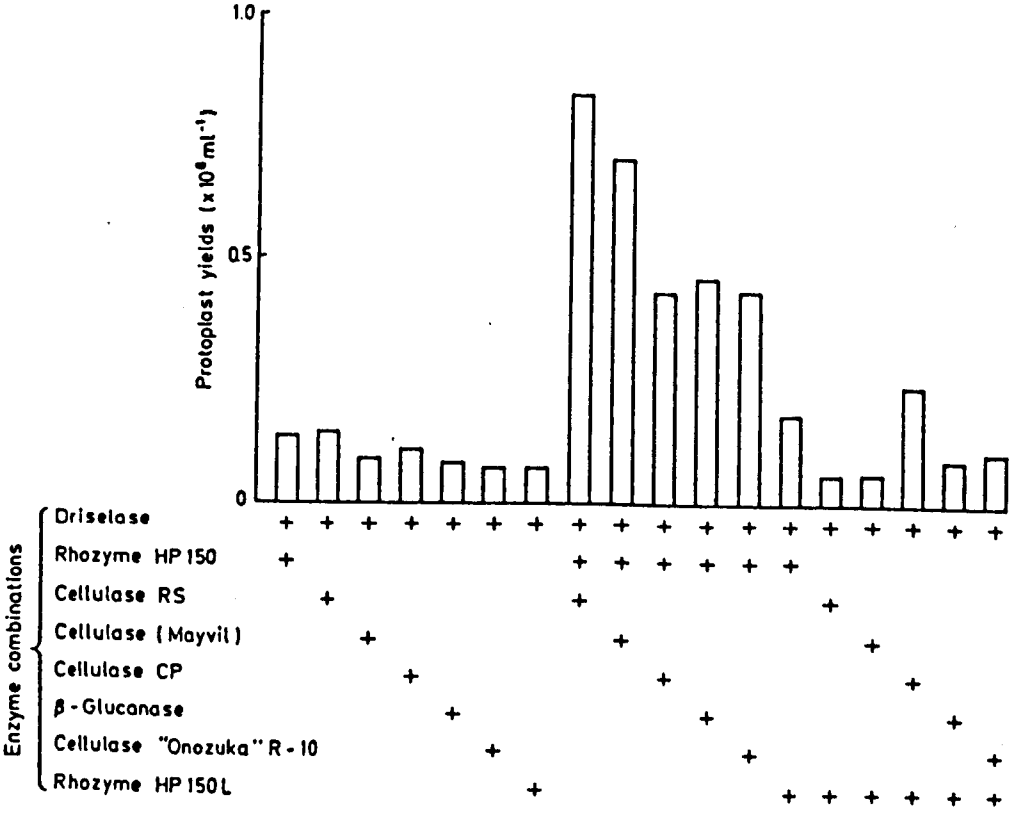
5.3.1 Protoplast Formation

5.3.1.1 Comparison of Enzyme Mixtures

Eighteen commercially available polysaccharase enzyme preparations were screened for their lytic activity against P. herpotrichoides. Novozym 234, found to have some lytic effect, was used in paired combinations with the other enzymes to screen for increased activity. These enzymes were also tested individually. Enzymes giving increased yields of protoplasts were then tested in novel combinations of two or three enzymes at a concentration of 5 mg ml^{-1} of each.

Cellulase (Boehringer), Cellulysin, Helicase, Lytic enzyme L1, Macerozyme R-10, Meicelase P, Pectinase (Serva), Pectolyase, Rolament P and snail enzyme (Szeged) were ineffective for protoplast release either singly or in combination. The most active mixtures were those that included Driselase and Rhozyme HP150. The addition of a third enzyme to a combination of these two enhanced protoplast release if the extra enzyme was one of the following: Cellulase RS, Cellulase (Mayvil), Cellulase CP, Cellulase "onozuka" R-10 or β-Glucanase (Fig. 5.1). Rhozyme HP150 was essential for the production of large numbers of protoplasts. A liquid form of this enzyme, Rhozyme HP150L, was not as effective. A combination of Driselase, Rhozyme HP150 and Cellulase CP was used as the standard enzyme mixture for the subsequent evaluation of other factors affecting protoplast release. This combination of enzymes has been used repeatedly,

Figure 5.1 Comparison of lytic activity of combinations of various commercial enzymes, against P. herpotrichoides. Protoplast numbers determined after 3 h incubation at 28°C. Enzymes dissolved in buffer containing KCl.



yields of protoplasts consistently being of the same order as given in the data presented.

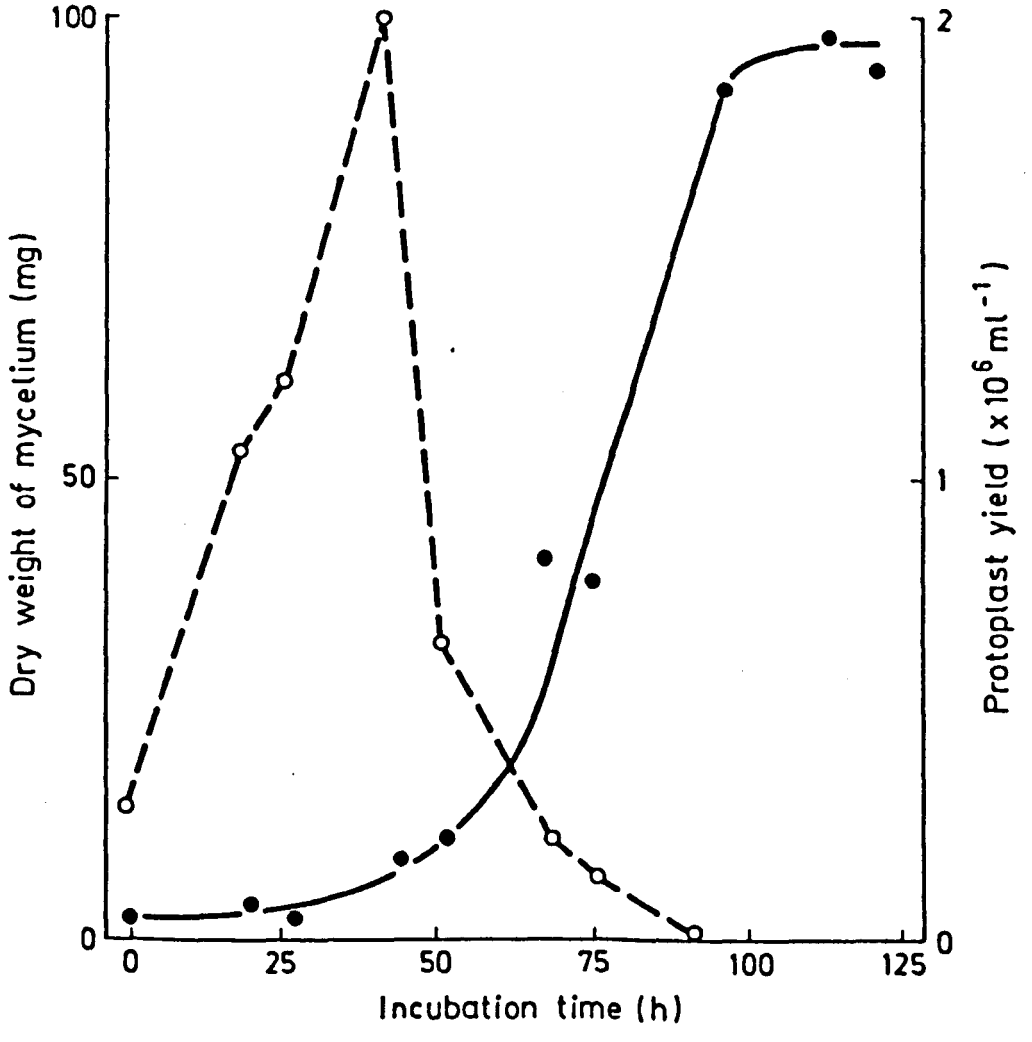
5.3.1.2 Mycelial Age

To determine the optimum age of mycelium for protoplast release, shake cultures inoculated with 10^5 spores ml^{-1} were harvested at intervals up to 120 h incubation, and the wet and dry weights of the mycelium measured. Mycelium recovered at each time interval was assessed for susceptibility to protoplast release by resuspending at 10 mg fresh weight ml^{-1} in the standard enzyme mixture. Fig. 5.2 shows the increase in dry weight of the mycelium during the experiment and the yield of protoplasts obtained at each stage. Maximum yields were achieved with mycelium grown for 44 h, which coincided with the early exponential phase of growth. Protoplast release from mycelium cultured for longer than 44 h was much reduced, illustrating the importance of the physiological age of the mycelium for protoplast isolation. This pattern of protoplast formation is a common feature of protoplast release in fungi, possibly reflecting changes in cell wall composition (Peberdy, 1979).

5.3.1.3 Osmotic Stabilizer

Five inorganic salts (NH_4Cl, $(NH_4)_2SO_4$, $MgCl_2$, $MgSO_4$, KCl, 0.4 to 0.8 M) were compared for their efficacy as osmotic stabilizers for protoplast release. The number of protoplasts liberated by each enzyme-stabilizer solution was determined after 6 and 22 h incubation. Large numbers of protoplasts were obtained with 0.4 M $MgCl_2$ and 0.8 M KCl (Fig. 5.3). In both instances

Figure 5.2 Protoplast release from mycelium of *P. herpotrichoides* at different stages of growth. ● = dry weight of mycelium; ○ = protoplast yield.



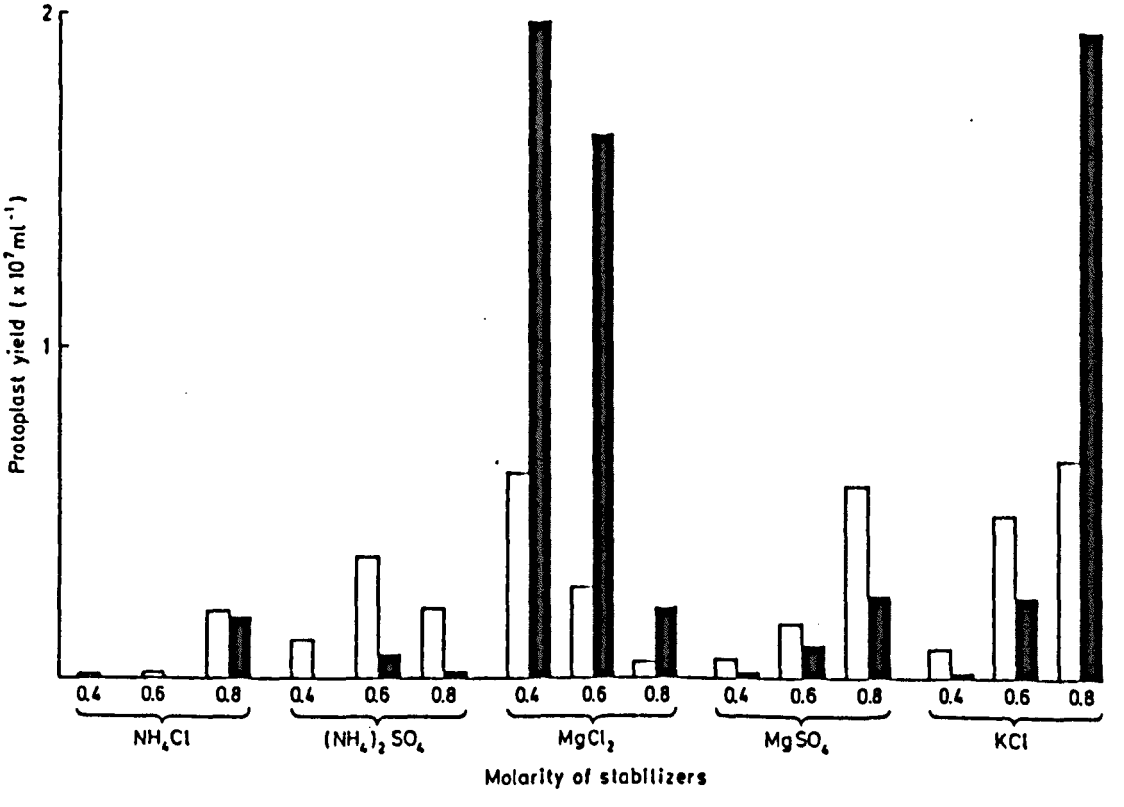
protoplast yields increased three-fold between 6 and 22 h incubation in the lytic mixture, reaching a maximum of almost 2 x 10^7 protoplasts ml^{-1}. When magnesium sulphate was used as the osmotic stabilizer the protoplasts released showed the characteristic buoyancy associated with this salt (Peberdy, 1979). Neither $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ or NH_4Cl were suitable as stabilizers for protoplast support with this species. Yields of protoplasts with all three salts were much reduced by 22 h incubation.

5.3.1.4 Enzyme Concentration

The yield of protoplasts obtained with 0.4 M $MgCl_2$ or 0.8 M KCl could be increased further by raising the amount of mycelium in the lytic mixture to 50 mg (fresh weight) ml^{-1} (Table 5.2). However, the incubation time required to obtain these high yields was considerable. In addition, protoplasts recovered after

24 h incubation in the enzyme solution had reduced viability when permitted to regenerate on osmotically stabilized MYG. In an attempt to reduce the incubation period the effect of adjusting the relative proportions and concentrations of the three enzymes in the lytic mixture was assessed (Table 5.3). Used singly, both Driselase and Cellulase CP produced significant numbers of protoplasts after 3h incubation, the number of protoplasts released increasing with enzyme concentration. Rhozyme HP150, while inactive against P. herpotrichoides mycelium when used alone, greatly increased the yields of protoplasts produced with

Figure 5.3 Effect of osmotic stabilizer on the yield of protoplasts from mycelium of P. herpotrichoides using the standard enzyme mixture. □ = lytic digests incubated for 6 h; ■ = lytic digests incubated for 22 h.



either of the other two enzymes. In combination with Driselase

Table 5.2 Effect of mycelium concentration on protoplast isolation with two osmotic stabilizers (Protoplast numbers ± standard error).

OSMOTIC STABILIZER	MYCELIUM CONCENTRATION (mg ml^{-1})	PROTOPLAST YIELD (x 10^6 ml^{-1})* AFTER 3 h INCUBATION	AFTER 24 h INCUBATION
0.4M MgCl2	10	0.5 ± 0.1	2.9 ± 0.2
	30	5.9 ± 0.4	8.1 ± 0.5
	50	8.2 ± 0.4	36.3 ± 1.2
0.8M KCl	10	3.1 ± 0.2	4.2 ± 0.3
	30	6.4 ± 0.2	3.5 ± 0.1
	50	11.0 ± 0.5	30.1 ± 1.3

* Mycelium grown for 42 h in shake culture, protoplasts produced in enzyme mixture containing Rhozyme HP150 + Driselase + Cellulase CP (5 mg ml^{-1} each).

the number of protoplasts obtained was increased 7- to 10-fold compared with the yields for Driselase alone. Similarly the yields when Rhozyme HP150 and Cellulase CP were used together were 4 to 14 times greater than those obtained with Cellulase CP alone. Clearly Rhozyme HP150 must contribute activities which complement those of the other components of the mixture. Increasing the concentration of all the enzymes in the lytic mixture to 10 mg ml^{-1} resulted in a doubling of the protoplast yield (Table 5.3). However, only a small increase was obtained when each enzyme was increased to 15 mg ml^{-1}.

Table 5.3 Protoplast yields with different combinations of Rhozyme HP150, Driselase and Cellulase CP. Protoplast numbers determined after 3 h incubation in the digestion mixture.

ENZYME SYSTEM	ENZYME CONCENTRATION (mg ml^{-1})	PROTOPLAST YIELD (x 10^6 ml^{-1}) (mean ± standard error)
Rhozyme HP150]	5	0
	10	0
	15	0
Driselase	5	0.52 ± 0.03
	10	1.82 ± 0.27
	15	5.49 ± 0.34
Cellulase CP	5	0.28 ± 0.02
	10	2.05 ± 0.16
	15	4.61 ± 0.40
Rhozyme HP150 + Driselase]	10 + 5	5.11 ± 0.62
	5 + 10	12.59 ± 1.04
Rhozyme HP150 + Cellulase CP	10 + 5	4.07 ± 0.29
	5 + 10	8.67 ± 0.49
Rhozyme HP150 + Driselase + Cellulase CP	5 + 5 + 5	22.29 ± 0.92
	10 + 10 + 10	44.00 ± 2.36
	15 + 15 + 15	48.38 ± 2.51

5.3.1.5 Enzyme Activities

To help interpret the results presented in Table 5.3 specific hydrolytic activities of the three enzyme preparations were assayed (Table 5.4). Chitinase and α- and β-D-glucanase activities were determined both for the individual enzymes and for all possible combinations of them. D-mannanase and protease activities were determined only for the individual enzymes. The pattern of activities exhibited varied considerably between the enzymes. The hydrolytic activities shown by enzyme mixtures do little to explain the enhancement of protoplast release observed with these enzyme combinations. Of the three main enzyme activities determined only the α-D-glucanase component of Cellulase CP detected against mutan, was increased by combination with the other enzymes. β-D-glucanase activities for enzyme mixtures were similar to those estimated from the values for the individual enzymes, suggesting an absence of interaction between the enzymes for this component. Chitinase activities determined for mixtures of the enzymes were generally much less than expected from the individual levels of activity. This reduction may be due to the presence of chitinase inhibitors in one or more of the enzymes, or possibly the result of proteolytic activity against the chitinase component of Cellulase CP. Both Driselase and Rhozyme HP150 had significantly higher levels of protease than Cellulase CP.

Table 5.4 Comparison of selected enzyme activities shown by different combinations of the lytic enzymes, Rhozyme HP150, Driselase and Cellulase CP. (mean ± standard error)

ENZYME SYSTEM	PROTEIN ($\mu g\ ml^{-1}$ enzyme preparation)	$x10^{-3}$ CHITINASE SPECIFIC ACTIVITY ($\mu mol\ mg^{-1}$ protein min^{-1})	$x10^{-2}$ β-D-GLUCANASE (LAMINARIN) SPECIFIC ACTIVITY ($\mu mol\ mg^{-1}$ protein min^{-1})	$x10^{-2}$ α-D-GLUCANASE (NIGERAN) SPECIFIC ACTIVITY ($\mu mol\ mg^{-1}$ protein min^{-1})	$x10^{-2}$ β-D-GLUCANASE (MUTAN) SPECIFIC ACTIVITY ($\mu mol\ mg^{-1}$ protein min^{-1})	D-MANNANASE ACTIVITY ($\mu mol\ mg^{-1}$ min^{-1})	PROTEASE (equivalent to μg trypsin mg^{-1} enzyme)
RHOZYME HP150	311±4	5.11±0.08	4.72±0.11	(0)	(0)	(0)	83 8
DRISELASE	134±6	(0)	33.58±0.43	(0)	(0)	(0)	127 5
CELLULASE CP	545±12	24.39±0.44	72.48±0.69	(0)	0.55±0.08	(0)	19 3
RHOZYME + DRISELASE (calculated)	222	5.09±0.05 (3.69)*	11.56±0.30 (13.51)	(0)	(0) (0)	-	-
RHOZYME + CELLULASE CP (calculated)	428	2.62±0.13 (17.38)	40.73±0.34 (47.90)	(0)	0.57±0.02 (0.35)	-	-

Table 5.4 continued

DRISELASE + CELLULASE CP (calculated)	340	3.82± 0.16 (19.62)	52.45±0.20 (64.71)	(0)	0.57±0.04 (0.44)	-	-
RHOZYME + DRISELASE + CELLULASE CP (calculated)	330	2.61±0.13 (15.06)	45.56±0.10 (46.10)	(0)	1.16±0.11 (0.30)	-	-

(0) No activity detected under the conditions of assay

- Not tested

* Estimated activities for combinations of enzymes, calculated from values for the individual enzymes, shown in parenthesis below actual activities.

5.3.2 Protoplast Regeneration

The ability of protoplasts to regenerate a cell wall and form normal colonies was initially assessed by plating on MYG agar medium containing either 0.4 M $MgCl_2$ or 0.8 M KCl. Regeneration on medium stabilised with KCl was very low (<2%); on medium containing magnesium chloride as the osmotic stabiliser, only 15 to 20% of the protoplasts formed viable colonies. To increase the plating efficiency several compounds were compared for their suitability as osmotic stabilisers in the regeneration medium (Table 5.5). When the osmoticum was a sugar or sugar alcohol, glucose was omitted from the medium. Very rapid regeneration occurred in the presence of sucrose, visible colonies appearing within just 3 days on both complete and minimal media (Fig.5.4a). On media stabilised by sodium chloride or magnesium chloride reasonable regeneration frequencies were obtained but a prolonged incubation period (3 to 4 weeks) was required, particularly for protoplasts cultured on minimal medium (Fig 5.4b). Sorbose and mannitol were unsuitable osmotica for high frequency protoplast regeneration.

Table 5.5 Regeneration frequency of protoplasts on complete (CM) and minimal (MM) medium in the presence of different osmotic stabilizers. Protoplasts were obtained after 3 h incubation in an enzyme mixture containing Rhozyme HP150 + Driselase + Cellulase CP (10 mg ml^{-1} each), stabilized with 0.4M $MgCl_2$. Colony numbers were determined after 4 to 28 days incubation at 19°C.

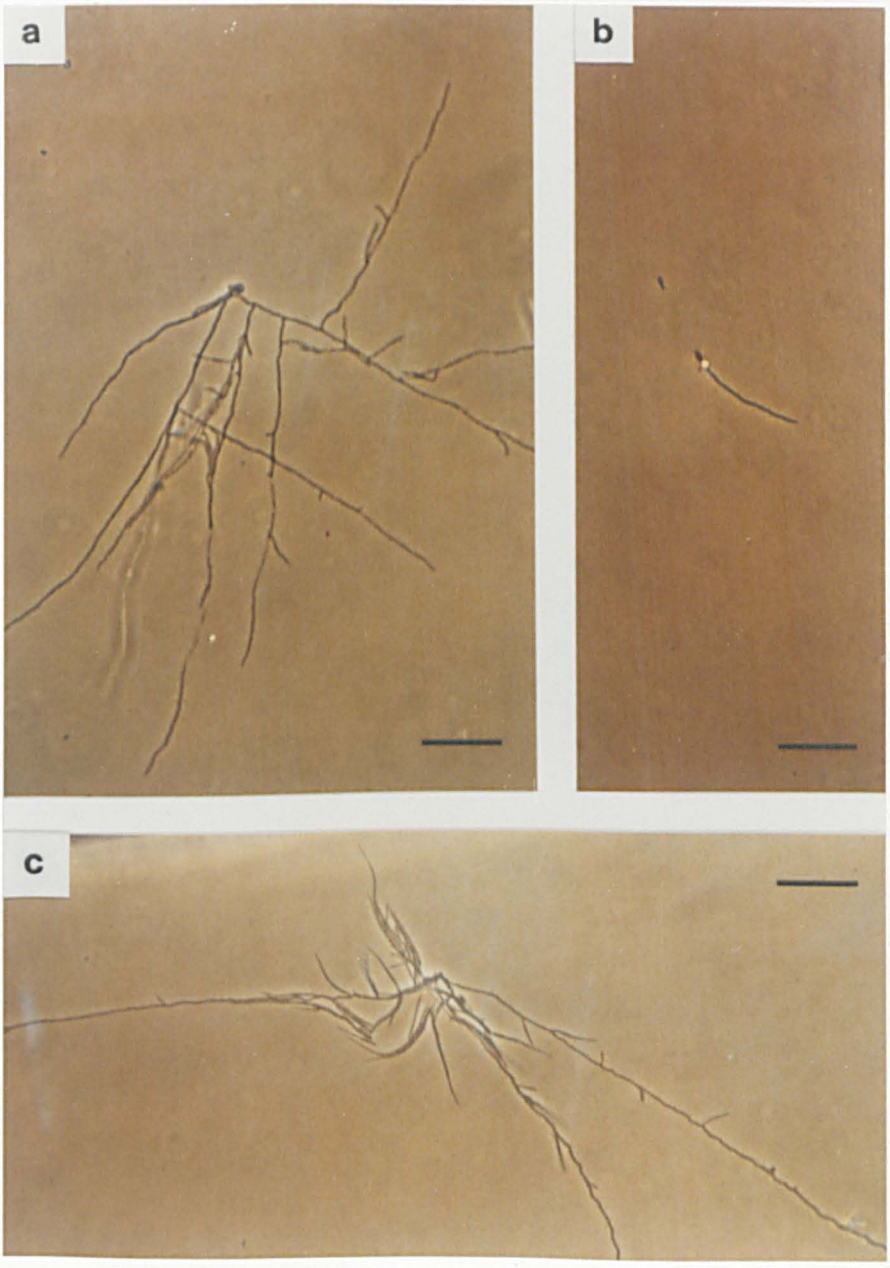
		REGENERATION FREQUENCY (% ± SE)	
STABILIZER	MOLARITY	CM	MM
$MgCl_2$	0.4	50.6 ± 2.1	38.8 ± 6.8
	0.6	44.4 ± 2.4	53.6 ± 1.3
	0.8	3.6 ± 2.5	0.0
SUCROSE	0.4	26.0 ± 1.1	40.5 ± 1.3
	0.6	43.8 ± 4.1	46.2 ± 0.3
	0.8	57.5 ± 3.8	43.9 ± 0.5
NaCl	0.4	38.5 ± 2.5	65.8 ± 4.0
	0.6	45.6 ± 2.4	63.3 ± 2.2
	0.8	22.2 ± 0.9	54.1 ± 3.4
	1.0	0.0	0.0
SORBOSE	0.4	0.0	0.0
	0.6	0.2 ± 0.1	0.0
	0.8	0.7 ± 0.2	6.2 ± 2.0
	1.0	1.2 ± 0.4	4.8 ± 1.1
MANNITOL	0.4	0.0	0.0
	0.6	0.0	0.8 ± 0.2
	0.8	3.6 ± 1.3	10.6 ± 3.4

Figure 5.4 Protoplast regeneration in isolate 22-20 (Bars represent 75 μm).

a Regenerant after 3 days on MMR stabilized with 0.6 M sucrose.

b Regenerant after 3 days on MMR stabilized with 0.4 M $MgCl_2$.

c Spore formation by 3 day old protoplast regenerant on MMR stabilized with 0.6 M sucrose.


a
b
c

5.4 DISCUSSION

The use of commercial polysaccharase enzymes for protoplast isolation from filamentous fungi is now commonplace (Davis, 1985). While most of these enzyme preparations consist of a mixture of several hydrolytic components, they have usually been used in combinations of two or three to achieve the optimum effect (Hamlyn *et al*, 1981; Harris, 1982; Hashiba & Yamada, 1982; Leslie, 1983; Yabuki *et al*, 1984; Yanagi *et al*, 1985).

A mixture of three lytic preparations Rhozyme HP150, Driselase and Cellulase CP was found to be the most effective for *P. herpotrichoides*. Cellulase CP has been widely used for protoplast isolation from filamentous fungi, often in combination with Novozym 234 (Bradshaw *et al*, 1983). Driselase, an enzyme regularly used to isolate plant protoplasts (Davey, 1983), has been successfully used for protoplast isolation from a few fungal species (Hashida & Yamada, 1982; Schafrick & Horgen, 1978). However, this enzyme could not be used in place of Cellulase "Onozuka" R-10 for the isolation of *Ustilago maydis* protoplasts (de Waard, 1976). Rhozyme HP150, also used routinely for plant protoplast production, has not apparently been used previously to produce fungal protoplasts. It is interesting that this enzyme was only effective when used in combination with other enzymes. The reason for this is not clear but presumably relates to additional side activities not tested in the enzyme assays described above. Rhozyme HP150 has been marketed commercially for the hydrolysis of vegetable gums and mucilages. Since the hyphae

of P. herpotrichoides are known to be ensheathed in mucilage (Reiss, 1971),the stimulatory effect of this enzyme for protoplast release may relate to the removal of this mucilage layer, permitting a greater accessibility of cell wall to the other enzymes in the preparation.

Chitinase activity was not essential for protoplast release in P. herpotrichoides, since protoplasts were obtained, although in reduced numbers, using Driselase alone. Chitinase activity could not be detected in this enzyme under the conditions of the assay. This observation suggests that chitin is absent or is only a minor component of the cell wall in this fungus, at least in the early stages of growth. It is reasonable to suppose that β-glucanase is a necessary component in the lytic mixture since all three enzymes used had significant levels of β-glucanase activity. The role of the α-glucanase component in the enzyme mixture is more difficult to assess since the detectable levels of activity were low, possibly as a result of poor solubility of the substrates used.

High frequency regeneration of protoplasts was obtained with three of the osmotica tested. Sucrose was the preferred stabiliser for regeneration since the growth rate of regenerant colonies was much greater with this than with either magnesium chloride or sodium chloride. In Cephalosporium acremonium sodium chloride was preferred to sucrose as the stabiliser for protoplast regeneration because auxotrophic strains were prone to cross feeding when sucrose was used (Hamlyn, 1982). Whether this is also true for P. herpotrichoides remains to be determined.

Regeneration usually involved the formation of hyphae directly from the protoplasts, rather than after repeated "budding", which was rarely observed. The conditions under which regeneration occurs may play a major role in determining the morphology of reversion to hyphal growth. In Rhizoctonia solani and Pyricularia oryzae direct production of a germ-tube by regenerant protoplasts occurred more frequently on solid media, while in liquid regeneration media the budding form of growth was more common (Hashiba & Yamada, 1982; Kobayashi et al, 1985). A proportion of the regenerating protoplasts from P. herpotrichoides did not give rise to mycelial colonies but underwent microcyclic conidiation (Fig. 5.4c). A similar phenomenon was described for regenerating protoplasts in Aspergillus awamori (Bobbitt & Douglas, 1982).

CHAPTER 6

RECOMBINATION STUDIES

6.1 INTRODUCTION

Prior to the early 1950's, genetic studies in fungi were restricted to those species with a perfect stage. The discovery and description of a parasexual cycle in Aspergillus nidulans made the genetic analysis of asexual species possible for the first time (Pontecorvo et al, 1953). Since then many reports of parasexual phenomena in a wide range of fungi have been published, suggesting that the system may be a feature common to all fungal groups (Pontecorvo, 1956; Pontecorvo & Kafer, 1958; Bradley,1962; Tinline & MacNeill, 1969; Caten, 1981).

The sequence of events occurring in A. nidulans, and shown to occur in several other species, is considered the standard parasexual cycle (Fig. 6.1) (Pontecorvo, 1956; Fincham et al, 1979; Hastie, 1981; Caten, 1981). Briefly, hyphal anastomosis (or protoplast fusion) between two strains establishes heterokaryosis with the presence of unlike nuclei in the same mycelium. Chance nuclear fusions, or karyogamy, may occur to produce diploid nuclei. If karyogamy occurs between unlike nuclei the resulting diploid nucleus will be heterozygous at all the loci for which the parental strains differed.

Two processes may take place in such nuclei to yield novel genotypes. In the first, mitotic recombination (Fig. 6.2), crossing-over occurs between homologous, non-sister chromatids. The subsequent migration of the recombinant chromatids to the same or opposite poles at mitosis, leads to daughter nuclei that

are either homozygous for all loci distal to the point of cross-over, or heterozygous but with altered linkage relationships for the parental markers. In the latter case the incidence of mitotic recombination will only be apparent after haploidization and analysis of the haploid genotypes. In the former, all recessive markers distal to the point of recombination will be expressed in the diploid. Consequently, the position of the cross-over relative to the markers on the same chromosome arm can be readily determined by examining the phenotype of the recombinant diploid (Pontecorvo et al, 1953; Pontecorvo & Kafer, 1958).

The second process leading to the formation of new genotypes is non-disjunction, in which mitotic division is unequal. Sister chromatids, of one or more chromosomes, both pass to the same pole and are included in the same nucleus. As a result, one of the daughter nuclei contains three copies of the chromosome and is hyperdiploid (2n + 1), while the other contains only one and consequently hypodiploid (2n - 1). In subsequent nuclear divisions the additional chromosome in hyperdiploid nuclei may be lost, regenerating the diploid chromosome complement; which is generally more stable than the aneuploid state. Depending upon which two of the three copies of the trisomic chromosome are retained the resulting diploid nucleus may be either identical to the original diploid, and heterozygous for all chromosomes, or homozygous for the linkage group in question (Fig. 6.3). In this latter case any recessive markers carried on this chomosome would be expressed.

Non-disjunctional nuclei containing less than the full diploid complement (ie. 2n - 1, 2n - 2, etc.), may yield haploid

Figure 6.1 The standard parasexual cycle in Aspergillus nidulans. Plasmogamy, or hyphal fusion, between different parental strains (● ○) produces a heterokaryotic mycelium in which heterozygous diploid nuclei (◑) may arise by random nuclear fusion. Recombinant diploids (◓) can be produced by mitotic crossing-over, while non-disjunction can yield recombinant haploids (⊜⊜) and diploids (⊕) as well as aneuploid types (⊜).

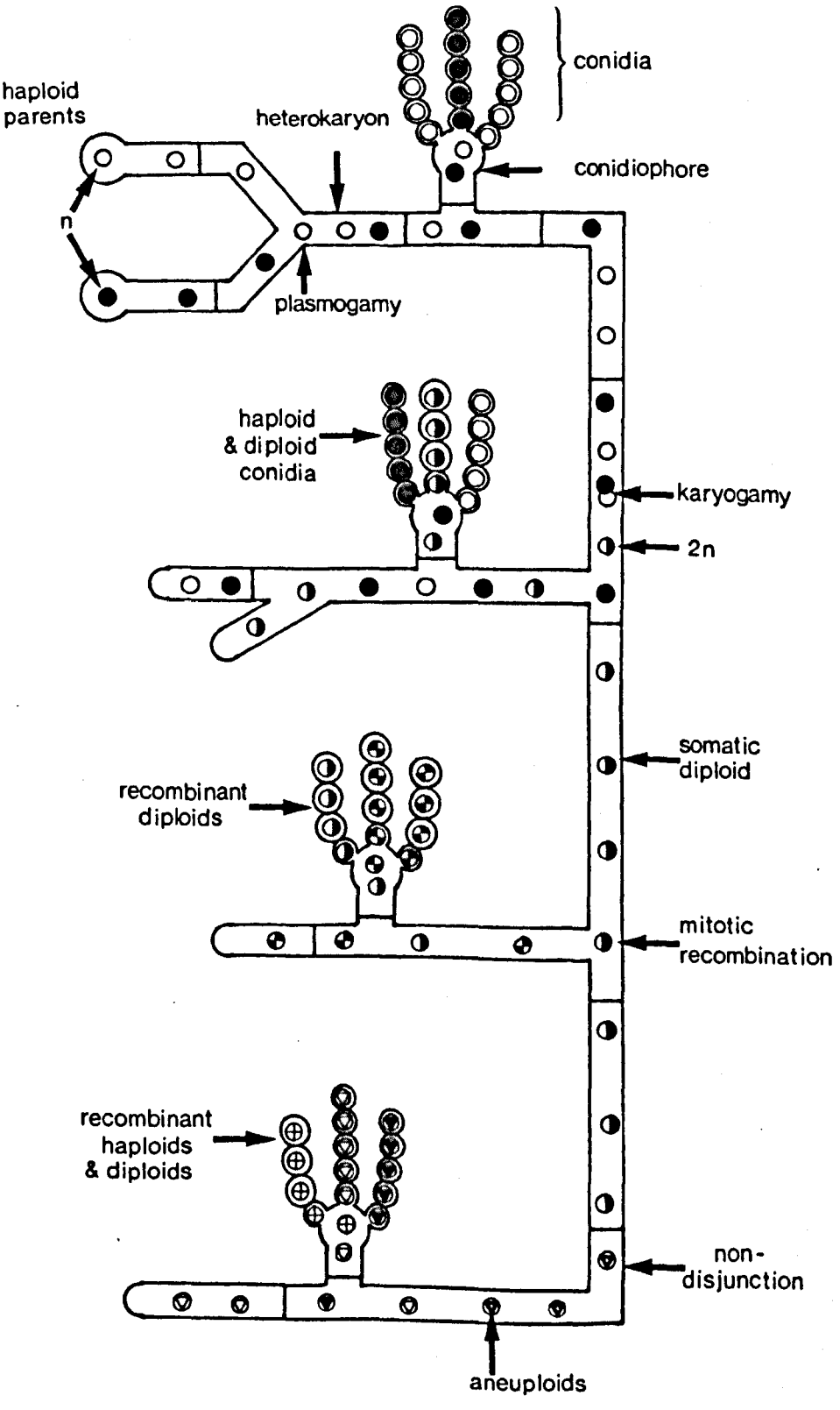

conidia
haploid
parents
heterokaryon
conidiophore
n
plasmogamy
haploid
& diploid
conidia
karyogamy
2n
somatic
diploid
recombinant
diploids
mitotic
recombination
recombinant
haploids
& diploids
non-
disjunction
aneuploids

Figure 6.2 Mitotic recombination in Aspergillus nidulans: cross-over between homologous non-sister chromatids results in novel linkage relationships. Depending on the subsequent migration of chromatids during division, the daughter nuclei may be homozygous for all markers distal to the point of cross-over; any recessive alleles carried in that region will consequently be expressed in the recombinant diploid.

Figure 6.3 Non-disjunction in Aspergillus nidulans: failure of sister chromatids to migrate to opposite poles during mitosis in a diploid nucleus produces aneuploid daughter nuclei. Sequential loss of additional chromosomes, or haploidization, in hypodiploid nuclei (2n-1) ultimately yield stable haploids. A second non-disjuctional event in the hyperdiploid nucleus (2n+1) may generate recombinant diploids as well as nuclei with higher ploidy levels.

Fig 6.2

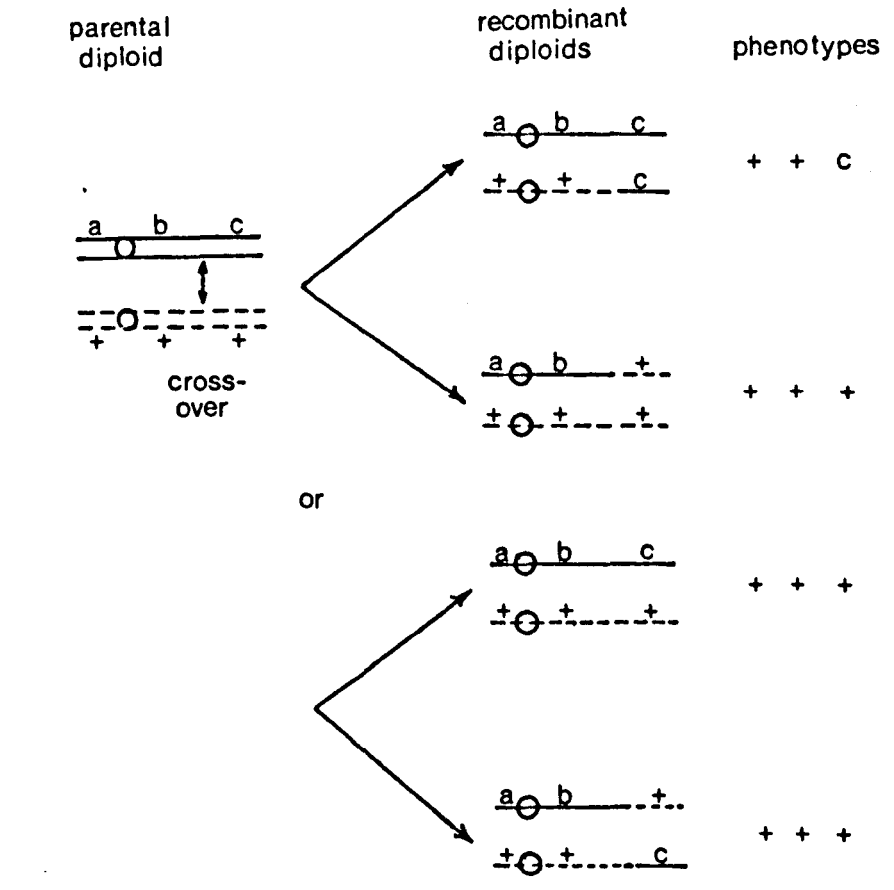
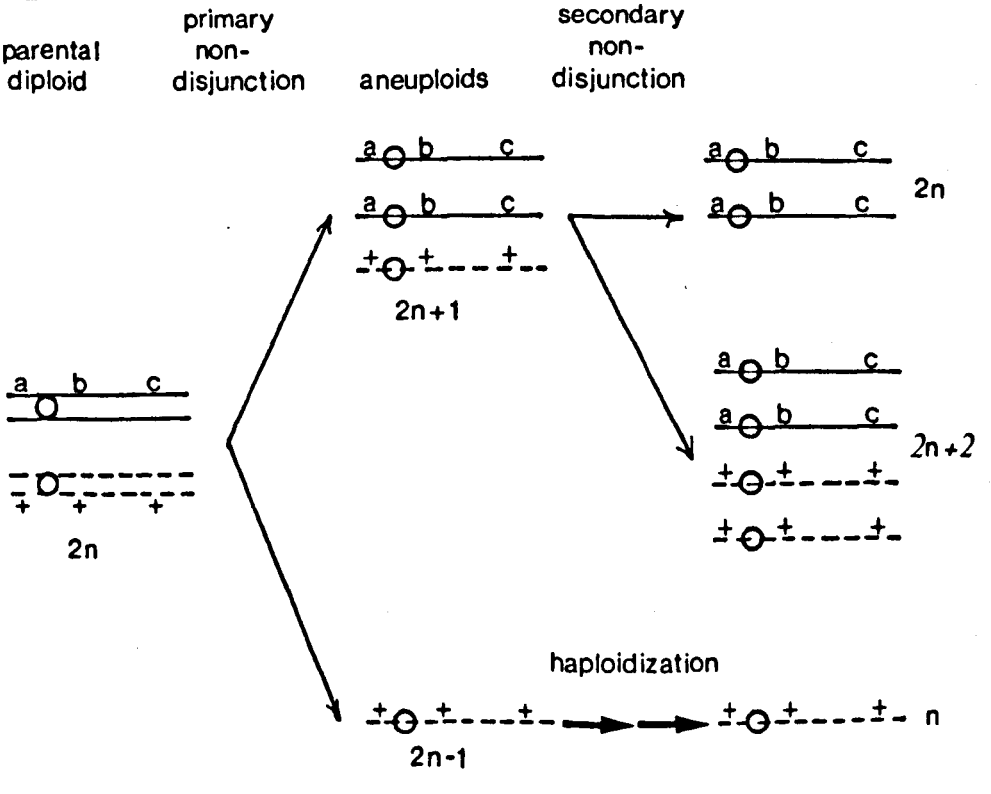

parental diploid
recombinant diploids
phenotypes
a b c
cross-over
or

Fig 6.3


parental diploid
primary non-disjunction
aneuploids
secondary non-disjunction
2n
2n+1
2n+2
haploidization
2n-1
n

nuclei as a result of the progressive loss of additional chromosomes: a process called haploidization (Fig. 6.3). Since this loss is generally a random process the resultant haploid nuclei will contain genetic material derived from either parent.

As might be expected, not all fungal species conform exactly to this format. Variations in the extent and duration of each stage occur, which have consequences not only for the manipulation of the process in the laboratory, but also for the likely occurrence of the cycle in the natural environment.

The first stage in the cycle, heterokaryon formation, is considerably influenced by the cytology of the fungus. Species in which the mycelium is composed primarily of multinucleate cells, such as A. nidulans, can readily accommodate the heterokaryotic condition. The majority of the vegetative cells will contain nuclei of both parents, and the relative proportions of the different nuclei will generally be stable and characteristic of the heterokaryon. Where conidia produced by such heterokaryons are multinucleate, as in A. sojae, the heterokaryon may be transmitted via the spores (Bradley, 1962). In A. nidulans, however, the conidia are uninucleate. Consequently, unless karyogamy has produced diploid nuclei which can be included in the conidia, plating spores from a heterokaryon of this fungus will produce only parental types.

Species in which uninucleate vegetative cells predominate may still show heterokaryotic growth, as a result of hyphal anastomosis forming bridges between individual cells. Nuclear migration may occur along the bridge to yield isolated,

heterokaryotic cells embedded in a mycelium of monokaryotic cells. The term "mosaic heterokaryon" has been used to describe this type of growth, typified by heterokaryons in Verticillium dahliae (Puhalla & Mayfield, 1974) and V. albo-atrum (Typas & Heale, 1976a). Where the parental strains carry complementary nutritional requirements, this limited degree of heterokaryosis is often sufficient, under selective conditions, to support the growth of the surrounding homokaryotic mycelium. Conidia from such heterokaryons will generally have been derived from uninucleate cells, and hence of parental type (Hastie & Heale, 1984). Mosaic heterokaryons are maintained by the formation of new hyphal bridges and their growth is dependent on the rate at which this process occurs. In V. dahliae hyphal anastomoses are formed relatively frequently at 21°C, but rarely at 30°C (Puhalla & Mayfield, 1974). As a result, heterokaryotic growth ceases when colonies are transferred to the higher temperature. Growth of diploid mycelium, produced by the fusion of nuclei in anastomosed cells, is not dependent upon temperature. Consequently, diploid mycelium may be isolated directly, as faster growing sectors, from the edge of heterokaryotic colonies, incubated at the restrictive temperature.

Heterokaryosis appears to be even more restricted in a Humicola sp., where somatic diploids were obtained directly from mixed cultures of auxotrophic strains (De Bertoldi & Caten, 1975). The heterokaryotic phase was assumed to be confined to the original fused cell, further growth being dependent upon nuclear fusion and the formation of a heterozygous diploid. A similar type of heterokaryon organisation has been reported in Fusarium

moniliforme, although no diploids were recovered (Puhalla & Speith, 1983).

Diploid nuclei can arise in a number of ways: either by nuclear fusion or as a result of mitotic or meiotic malfunction. Mitotic malfunction, unless accompanied by mutation, will produce only homozygous diploids; phenotypically identical to the haploid in respect of auxotrophic and pigmentation markers. Such diploids can be distinguished from the haploid form on the basis of increased nuclear and cell volume, elevated DNA content and by their reduced sensitivity to mutagens such as UV-light (Caten & Day, 1977; Hastie & Heale, 1984). Homozygous diploid nuclei can also be produced by nuclear fusion and meiotic malfunction. In addition, these latter mechanisms have the potential to produce heterozygous somatic diploid nuclei, thereby enabling parasexual reassortment of the genetic material. In Neurospora crassa attempts to isolate somatic diploids or their recombinant products from heterokaryons have failed. Diploid ascospores, however, are occasionally produced by a failure of meiosis, and the products of mitotic crossing-over and haploidization can be isolated from the resulting mycelium.

In asexual species somatic nuclear fusion remains the main way in which heterozygous diploid nuclei can be generated. Estimation of the frequency with which nuclear fusions occur is technically difficult and is generally based on the frequency of isolation of diploid conidia from a heterokaryon. However, the recovery of diploid conidia will depend, amongst other factors, on the relative rates of division of diploid and haploid nuclei

and the degree of sporulation by diploid and haploid hyphae (Caten & Day, 1977; Caten, 1981). In addition, the stability of diploid nuclei will directly affect the frequency with which they are identified. The existence of diploid nuclei is often inferred from the occurrence of parasexual recombinants in the spore population, rather than from the direct isolation of the diploid itself. In Cephalosporium acremonium, for example, the diploid stage is presumed to be transient: recombinant haploid and unstable heterozygotes are obtained by plating heterokaryons directly onto selective media (Nuesch et al, 1973; Hamlyn & Ball, 1979; Hamlyn et al, 1985).

The frequency of diploid formation in heterokaryons may be affected by various factors, including heat treatment (Alikhanian et al, 1960), UV-irradiation (Day & Jones, 1968; Clements et al, 1969; Day & Day, 1974; Caten & Day, 1977), camphor treatment (Pontecorvo et al, 1953; Ishitani et al, 1956; Day & Day, 1974), incubation temperature and carbon-source (Ingle & Hastie, 1974).

The frequency of mitotic recombination or crossing-over also varies between species (Day, 1971). In A. nidulans the frequency is about 0.03 per genome per division (Kafer, 1977). In other species the frequency is much higher- >0.4 in V. albo-atrum (Hastie, 1967). The frequency of crossing-over is not uniform along the length of the chromosome. In A. nidulans the occurrence of both a sexual and a parasexual cycle allows the frequency of cross-over between linked markers during meiosis and mitosis to be compared (Pontecorvo & Kafer, 1958; Kafer, 1977). Considerable differences in mitotic and meiotic map distances were found. In mitotic recombination most cross-over events occurred close to

the centromere, presumably reflecting a close association of centromeres between homologous chomosomes in somatic diploids and little association between the distal ends of homologous chromatids. Similar observations have been made for Ustilago violacea (Garber & Day, 1985). Little meiotic recombination occurs between the markers and centromere for either the mating-type locus (Linkage group VI), or the colour mutation complex (Linkage group VII). By contrast, spontaneous mitotic crossing-over in these intervals occcurs at high frequency (Garber & Day, 1985).

A number of agents have been shown to increase the frequency of mitotic cross-overs. These include UV-irradiation (Holliday, 1961; Day & Jones, 1968; Wood & Kafer, 1969; Zimmerman, 1971; Tanabe & Garber, 1980; Amirkhanian et al, 1985), heat treatment (Tanabe & Garber, 1980) and chemical "recombinogens" such as benomyl, 5-fluorodeoxyuridine, 5-fluorouracil, sulphur mustard, nitrogen mustard, ethyl methane sulphonate and mitomycin C (Holliday, 1964; Eposito & Holliday, 1964; Beccari et al, 1967; Yost et al, 1967; Hastie, 1970; Tanabe & Garber, 1980; Kokontis & Garber, 1983). Since many of these are likely to cause damage to the DNA it is thought that they induce crossing-over by stimulating the DNA-repair mechanisms of the cell.

Haploidization can also be artificially induced by both chemical and physical agents (Barron, 1962; Bignami et al, 1974; Kafer et al, 1986). The haploidizing affect of p-fluorophenylalanine (FPA) and the benzimidazole fungicide benomyl

have long been recognised (Morpugo, 1961; Lhoas, 1961; Hastie, 1970) and are routinely used to induce haploidization of diploids for genetic analysis (McCully & Forbes, 1965; Fincham *et al*, 1979). Benomyl, or rather its breakdown product carbendazim, is known to bind to β-tubulin, and consequently interferes with normal microtubule formation and function (Davidse & Flach, 1977). Disruption of the mitotic spindle would be expected to increase the frequency of non-disjunction and hence the production of aneuploid nuclei. As some benomyl-resistant mutants in *A. nidulans* are cross-resistant to FPA, and FPA has been shown to bind to tubulin, it is highly likely that FPA works in the same way as benomyl (Morris & Oakley, 1979).

Aneuploid colonies are generally considered to be mitotically unstable, generating faster-growing, euploid sectors at high frequency (Kafer, 1960; Day & Jones, 1971). However, the stability of particular aneuploids may vary considerably. Of the possible monosomic and trisomic aneuploids in *A. nidulans* those involving chromosomes IV, VI and VII are the most stable, and produce colonies with large central aneuploid regions. Aneuploids involving chromosomes II or VIII on the other hand are much less viable, forming very slow growing and unstable colonies (Kafer & Upshall, 1973). Interestingly each of the aneuploids had a different, characteristic phenotype, independent of the particular markers present (Upshall, 1971). Genes affecting aneuploid stability at elevated temperatures have been identified in *A. nidulans*. One class of mutations (*hfa*; high frequency of aneuploids) generated nuclei aneuploid for several linkage groups at high frequency at the sub-restrictive temperature of 37°C. The

second class of mutations (sod; stabilization of disomy) allowed the stable maintenance of colonies disomic for one particular chromosome at the elevated temperature (Upshall & Mortimore, 1984; O'Donnell et al, 1986).

Differential stability of particular aneuploids has also been described in Ustilago violacea (Day & Jones, 1971). Haploidization of heterozygous diploids using p-fluorophenylalanine yielded only 1-5% haploids. Approximately 30% of the rest of the colonies were stable aneuploids, monosomic for one of two linkage groups (IX or X). In this instance aneuploidy was associated with mutation to resistance to the haploidizing agent (Day & Jones, 1971; Garber & Day, 1985). In Bremia lactucae , secondary homothallic strains were found to be trisomic for one chromosome, associated with, and presumably stabilized by, a reciprocal translocation (Michelmore & Ingram, 1982).

Parasexual recombination has been demonstrated in many different species of fungi under laboratory conditions (Day & Jones, 1968; Tinline & McNeill, 1969; Hastie, 1981). In most instances complementary nutritional requirements have been used to select forcibly for the products of "hybridisation". Such systems impose an artificial selection for the processes of the parasexual cycle and do not necessarily show that equivalent events occur naturally in the absence of selection (Caten & Jinks, 1966). Neutral genetic markers, such as morphological or pigmentation differences have been used in an attempt to avoid this criticism (Grindle, 1963a, b; Puhalla, 1979, 1984a). Non-selective markers can reveal differences in heterokaryon

formation; combinations of strains that appear compatible using auxotrophic mutants may prove not to be when colour mutants are used (Hastie & Heale, 1984). The main difficulty in demonstrating parasexuality in the field is one of identification of the products of such processes: heterokaryons, diploids and recombinants in the absence of selection. However, naturally occurring, diploid strains of some species have been isolated in V. dahliae (Ingram, 1968), A. niger (Nga et al, 1975), V. albo-atrum , A. nidulans and with greater frequency from the smut fungi U. maydis and U. violacea (Caten & Day, 1977; Caten, 1981; Hastie & Heale, 1984). The diploid strains of V. dahliae and some of those of A. nidulans were homozygous, and therefore with limited potential for genetic reassortment. Those of A. niger, V. albo-atrum, and the remaining A. nidulans diploids were at least partially heterozygous, and segregated in the presence of a haploidizing agent. The widespread occurrence of incompatibility for heterokaryon formation in fungi further reduces the potential for genetic exchange in populations (Leach & Yoder, 1983; Hastie & Heale, 1984; Puhalla, 1984b, 1985; Clarkson & Heale, 1985a; Newton & Caten, 1985; Correll et al, 1986). Caten and Jinks (1966) have suggested that the role of parasexual recombination in wild populations may have been over-estimated by a failure to recognise the extent of heterokaryon incompatibility. Vegetative incompatibility in P. herpotrichoides will be considered in Chapter 7.

No detailed genetic studies have been undertaken with P. herpotrichoides. However, a diploid strain was synthesized from two independent, prototrophic isolates differing in pigmentation

by Davies and Jones (1970a). The diploid was identified on the basis of altered pigmentation and showed approximately twice the amount of DNA per nucleus compared with the parent strains. FPA was used to induce haploidization, and both parental types were recovered. Since no other recognisable characters were present in the parent isolates parasexual reassortment of markers could not be demonstrated, and no genetic analysis was possible. Interestingly the diploid had a faster growth rate than the parental isolates, on agar medium, suggesting a selective advantage for the diploid over its parent isolates. In pathogenicity tests the diploid caused disease symptoms in barley plants, although the severity of the symptoms was less than produced by either parent strain, possibly reflecting the diverse origin of these isolates (Davies & Jones, 1970b)

The following chapter describes attempts to demonstrate parasexual events in P. herpotrichoides, using genetically marked strains. Two techniques were used to obtain heterokaryons: orthodox procedures, involving hyphal anastomosis, and protoplast fusion. The latter technique has proved useful to produce heterokaryons between vegetatively incompatible isolates in other species (Dales & Croft, 1977; Typas, 1983; Zhemchuzhina et al, 1985).

6.2 MATERIALS AND METHODS

6.2.1 Strains

Strains were derived from isolates 22-20 (BW-type) and 22-12 (BWR-type). Gene symbols and the origin and characteristics of the auxotrophic mutants are described in Chapter 4.0. The mutants derived from 22-20 were as follows: 22-127 (lysB-4, ben-17);

22-133 (hisA-1, metB-2);

22-134 (hisA-1, uraA-1);

22-135 (hisA-1, sC-6);

22-136 (hisA-1, argB-3);

22-137 (hisB-2, ben-17).

22-139 (arg-A-1, red-1)

Strain 22-184 (con-2) was produced from isolate 22-12.

6.2.2 Orthodox Crosses

6.2.2.1 Heterokaryon Formation

Heterokaryons were established by hyphal anastomosis. Conidia of the parental strains were co-inoculated into 2-5 ml of liquid minimal medium (MML) supplemented with 0.6 mg ml^{-1} yeast extract, in screw-capped glass tubes (10^5 spores of each strain). After incubation for 19 days at 19°C, the resultant mycelial suspension was washed with sterile distilled water by centrifugation and resuspended in 5 ml water. One hundred µl aliquots of suspension were spread on plates of minimal agar (MM) with a sterile glass spreader. Ten plates were inoculated for each parent combination, with the exception of 22-137 x 22-184

for which twenty plates were used. Control suspensions of the individual parental strains were similarly treated.

Regions of dark-pigmented, prototrophic mycelium were visible after 16 days at 19°C. Prototrophic mycelium was picked off and point inoculated onto fresh MM. The growth of these colonies was visually assessed after 11 days.

6.2.2.2 Stability of Prototrophic Colonies

The stability of the prototrophic colonies was assessed in two ways. In the first, spores and hyphal fragments were scraped off the surface of the colonies growing on MM, suspended in sterile distilled water and plated onto MM and MYG. The number of colonies appearing on the two media was compared. In addition, samples of those colonies appearing on MYG were tested for auxotrophic requirements and carbendazim resistance by direct transfer onto appropriate diagnostic media.

The second method, by which prototroph stability was assessed, involved the culture of protoplasts on MMR and MYR. Agar blocks (approximately 1 mm^3) were cut from the prototrophic colonies growing on MM and placed on cellophane sheets over MYG or MM. Plates were incubated for 4 to 14 days at 19°C. For protoplast formation eight colonies, were excised and suspended in 1 ml of a lytic enzyme mixture containing Rhozyme HP150, Driselase and Cellulase CP in 0.05M sodium maleate buffer and 0.4 M MgCl2 as described in Chapter 5.0. After 3 to 4 h digestion the protoplasts were harvested by filtration through sinter glass (porosity 2) and recovered by centrifugation (10 min, 3000 x g). Protoplasts resuspended in osmotic stabilizer were plated onto

minimal (MMR) and complete (MYR) regeneration media to give between 150 and 210 protoplasts per plate. Lysed suspensions prepared in sterile water were also plated, to detect the presence of spores or hyphal fragments. As for the spore-derived material, samples of the colonies, growing on MYR, were tested for nutritional requirements and carbendazim resistance.

6.2.2.3 Induced Segregation and Isolation of Recombinants

Stable prototrophic colonies and parental types were point inoculated onto MYG containing either 100 μM carbendazim, 50 μM MDPC or 100 and 200 μg ml^{-1} 5-fluorouracil and allowed to grow for 21 days. On complete medium few conidia are produced, so mycelium from each colony was transferred to TWA containing the appropriate supplements. After 8 weeks, blocks of agar (approximately 1 cm^2) were excised and shaken in 5 ml of sterile distilled water to dislodge the conidia. Single drops of the spore suspensions were then plated onto MYG. The morphology, pigmentation, size and stability of the resulting colonies was visually assessed after 14 days. Samples were screened for auxotrophic and resistance markers as follows: master plates containing 16 colonies per plate were constructed on MYG. After 10 to 14 days the colonies were multipoint replicated onto the appropriate diagnostic media.

Slow-growing, unstable colonies were "purified" by macerating a small quantity of mycelium in water and plating the homogenate onto MYG. The colonies produced were characterised as described.

6.2.2.4 DNA Estimation

Colorimetric estimates of DNA were performed using the method of Richards (1974). As few spores were produced by the proto-trophic products of hyphal anastomosis, lysed protoplast preparations were used to provide the DNA samples. Protoplasts produced from mycelium grown on cellophanes over MYG were resuspended in 1 ml sterile distilled water with 37.5 Ml of 55% trichloroacetic acid. The DNA was hydrolysed for 10 min at 90°C and the sample cooled to room temperature. Insoluble material was removed by centrifugation at 11,600 x g for 10 min. To 0.75 ml of the supernatant was added 0.25 ml of 60 % perchloric acid and 0.6 ml of diphenylamine reagent (4% diphenylamine in glacial acetic acid containing 0.01% paraldehyde). Colouration was allowed to develop for 24 h at 28°C and the absorbance measured at 595 nm. A standard curve was constructed using calf thymus DNA.

6.2.2.5 Estimation of Number of Nuclei in Protoplasts

Protoplasts were fixed with glutaraldehyde. Minimal distortion and lysis was achieved by increasing the glutaraldehyde concentration in three stages (1.25%, 2.5%, 5%), allowing 20 minutes at each concentration. Nuclei were stained with chromomycin A_3 (Sigma; 0.4 mg ml^{-1} prepared in 50% aqueous ethanol containing 30 mM $MgCl_2$) on the slide and viewed under UV-fluorescence.

6.2.3 Protoplast Fusion

Protoplasts were prepared from mycelium grown in shake culture as decribed in Chapter 5.0. Fusion was performed

according to the protocol described by Hamlyn (1982), for Cephalosporium acremonium. Protoplasts (10^7 per strain) of each of the two parental strains were mixed and pelleted by centrifugation. The pellet was resuspended in 2 ml of 30% polyethylene glycol (PEG, MW 6000; Sigma) in 0.05 M glycine buffer containing 0.01 M $CaCl_2$ (adjusted to pH 7.5 with NaOH) and left for 10 min at room temperature. The PEG was diluted by the addition of 6 ml 0.4 M $MgCl_2$ and the mixture washed with osmoticum by centrifugation. Fused protoplasts were resuspended in 2 ml stabilizer and serial dilutions plated on MYR and MMR. Control platings of PEG-treated and untreated protoplasts of the individual parents were made, as well as untreated mixtures of the two strains.

Fusion frequency was calculated as the proportion of colonies appearing on MMR compared with MYR, after 12 days. Prototrophic regenerants on MMR were transferred to MM and treated in the same way as the products of the orthodox crosses described above.

6.4 Chemicals

Diphenylamine and trichloroacetic acid were purchased from Sigma Chemical Co. Calf thymus DNA, paraldehyde, perchloric acid and glacial acetic acid were from BDH Ltd., Poole.

6.3 RESULTS

6.3.1 Orthodox Crosses

6.3.1.1 Heterokaryon Formation

Conidia of the auxotrophic parental strains were co-inoculated into MML in the following combinations:

22-127 (*lysB-4*, *ben-17*) x 22-133 (*hisA-1*, *metB-2*)

22-127 (*lysB-4*, *ben-17*) x 22-134 (*hisA-1*, *uraA-1*)

22-127 (*lysB-4*, *ben-17*) x 22-135 (*hisA-1*, *sC-6*)

22-127 (*lysB-4*, *ben-17*) x 22-136 (*hisA-1*, *argB-3*)

22-133 (*hisA-1*, *metB-2*) x 22-139 (*argA-1*, *red-1*)

22-137 (*hisB-2*, *ben-17*) x 22-184 (*con-2*)

When mycelium from these cultures was plated onto MM areas of dark-pigmented, prototrophic growth were obtained. The number and appearance of these colonies is given in Table 6.1. Most colonies were slow-growing and irregular, while a proportion were faster-growing and had an even colony margin. In addition to dark-pigmented colonies the combination involving strain 22-134 also produced a number of slow-growing, non-pigmented colonies, which resembled the leaky growth obtained by plating this mutant separately on MM. Leaky, background growth was also observed for strain 22-139. Most of the growth on the MM plates inoculated with mycelium from the combination 22-137 x 22-184 was typical of strain 22-184, which formed small, red-brown, sporulating colonies on MM. However, a few non-sporing regions with profuse, grey, aerial mycelium were produced on these plates.

Table 6.1 Number of fast-even edged and slow-irregularly edged, dark pigmented, prototrophic colonies appearing on MM after plating mycelium from mixtures of auxotrophic strains. Plates incubated for 16 days at 19°C.

PARENT COMBINATION	NUMBER OF COLONIES ON MM FAST DARK EVEN	SLOW DARK IRREGULAR	TOTAL
22-127 x 22-133	13	36	49
22-127 x 22-134	3	45	48*
22-127 x 22-135	14	53	67
22-127 x 22-136	14	139	153
22-133 x 22-139	1	8	9*
22-137 x 22-184	0	4	4**
22-127	-	-	0
22-133	-	-	0
22-134	-	-	28***
22-135	-	-	0
22-136	-	-	0
22-137	-	-	0
22-139	-	-	220***
22-184	-	-	>>****

* Leaky background growth of one of the parental strain also observed.
** Considerable background growth of strain 22-184 (con-2) also produced.
*** All characteristic of leaky growth of this mutant.
**** All very small sporing colonies, typical of 22-184.

Prototrophic mycelium from three of the mutant combinations (22-127 x 22-133; 22-127 x 22-135; 22-137 x 22-184) was point inoculated onto fresh MM for further analysis. All 60 of the colonies from 22-127 x 22-135 and 43 of the 60 colonies from 22-127 x 22-133 tested were successfully transferred and formed colonies on MM. While all the prototrophs formed dark-pigmented colonies, some were slow and irregular while others were faster and had a regular margin. On prolonged incubation all colonies

eventually formed a relatively fast-growing and regular edge.

Of the 114 sub-cultures of the combination 22-137 x 22-184 on MM only 4 formed colonies that differed from those normally produced by the conidiating parent 22-184. These four colonies were relatively slow growing with abundant aerial mycelium.

6.3.1.2 Prototroph Stability

The stability of a number of the prototrophic colonies, differing in appearance on MM, was tested by plating spores and hyphal fragments onto complete and minimal media. The number of colonies obtained on the different media is presented in Table 6.2. Interestingly, conidia from those colonies producing the greatest proportion of stable prototrophs also showed the highest viability on MYG, though why this should be so is not clear.

A sample of colonies appearing on the MYG plates was characterised with respect to auxotrophic and resistance markers (Tables 6.3, 6.4). For each of the crosses 22-127 x 22-133 and 22-127 x 22-135 two of the three colonies on MM yielded predominantly auxotrophic colonies with the parental phenotypes. The third prototroph in each case produced a significant proportion of prototrophic, non-parental types.

Of the colonies tested from the 22-137 x 22-184 combination two gave colonies typical of the 22-184 parent, while the third yielded, in addition to 22-184-types, a number of non-parental colonies (Fig. 6.4).

The stability of prototrophs when propagated via protoplasts was also assessed (Table 6.5). Protoplasts were readily obtained

Table 6.2 Stability of prototrophic colonies: Proportion of spores, taken from prototrophic colonies growing on MM, producing colonies when plated on complete (MYG) and minimal (MM) media. Colonies were counted after 18 days at 19°C.

PARENTAL STRAINS	PROTOTROPH NUMBER	TOTAL SPORES PLATED	REISOLATION MEDIUM	NUMBER OF COLONIES PRODUCED	PROPORTION OF SPORES FORMING COLONIES (%)
22-127 x 22-133	1	224	MYG	60	27
		2600	MM	2	0.07
	2	200	MYG	144	72
		1100	MM	263	24
	3	280	MYG	82	29
		3220	MM	3	0.09
22-127 x 22-135	1	230	MYG	28	12
		45650	MM	1	0.002
	2	400	MYG	252	63
		600	MM	447	75
	3	230	MYG	44	19
		45600	MM	1	0.002
22-137 x 22-184	1	200	MYG	143	72
		300	MM	105	35
	2	200	MYG	149 (31)*	75 (16)
		300	MM	130 (43)	43 (14)
	3	260	MYG	198	76
		390	MM	191	49

* numbers in parentheses denote the number of dark-pigmented, non-sporing colonies produced. All other colonies derived from this cross were characteristic of the 22-184 (con-2) parent.

from mycelium of prototrophs from 22-127 x 22-133 and 22-127 x 22-135, grown on cellophanes over MYG, and regenerated with frequencies between 8 and 24% on MYR. As with the spore progeny, the number of colonies produced on minimal medium was variable. Only 12% of the viable protoplasts from three of the prototrophs regenerated as prototrophic colonies while the other three prototrophs tested gave between 36 and 89% prototrophic regenerants. The absence of colonies on MYR from platings of lysed protoplast suspensions indicated that none of the prototrophic colonies were derived from mycelial debris in the protoplast preparations. Samples of protoplsts from parental strains and prototrophs were fixed and the nuclei stained with chromomycin. Between 36% and 50% of the protoplasts were nucleate, the vast majority having a single nucleus.

The phenotypes of samples of the regenerant colonies produced on MYR are also presented in Tables 6.3 and 6.4. In each cross one of the three original prototrophic colonies tested gave a substantial proportion of prototrophic colonies from protoplasts. The remaining colonies yielded predominantly parental phenotypes. The preponderance of 22-127 parental types among the regenerant colonies from protoplasts produced from the cross with strain 22-133, was probably due to the lower viability of the latter strain, associated with the metB-2 mutation.

Due to the profuse sporulation of strains carrying the con-2 mutation it was not possible to produce protoplasts from the cross 22-137 x 22-184 without considerable contamination with conidia. Attempts to separate protoplasts from the conidia using

Table 6.3 Stability of prototrophic colonies: colony phenotypes produced from platings of spores and hyphal fragments or protoplasts of prototrophs derived from 22-127 (*lysB-4*, *ben-1*7) x 22-133 (*hisA-1*, *metB-2*) on MYG and MYR respectively.

SOURCE MATERIAL	PROTOTROPH NUMBER	NUMBER OF COLONIES IN EACH CLASS*				
		his$^-$ *met*$^-$	*lys*$^-$ *ben*^{R}	PROTOTROPHS	OTHER	TOTAL
Spores + hyphal fragments	1	14	33	2	2	51
	2	0	24	33	3	60
	3	0	59	1	0	60
Protoplasts	3	7	33	5	1	46
	4	0	40	19	1	69
	5	8	50	2	0	60

* *his*$^-$ = histidine-requiring; *met*$^-$ = methionine-requiring; *lys*$^-$ = lysine-requiring; *ben*^{R} = carbendazim-resistant.

0.6 M $MgSO_4$ were unsuccessful. Consequently no data for prototroph stability via protoplasts for this cross are available. Similarly insufficient protoplasts were obtained from all colonies grown on cellophane sheets laid over MM.

Studies were continued with stable prototrophs derived from the cross between 22-127 and 22-135. Several of these colonies, together with the parental strains are shown in Fig 6.5, growing on diagnostic media. The appearance of the stable prototrophs on MYG differs from either of the parents, having a yellow-brown pigmentation, while the colours of the parent strains 22-127 and 22-135 are black and grey, respectively.

Table 6.4 Stability of prototrophic colonies: colony phenotypes produced from platings of spores and hyphal fragments or protoplasts of prototrophs derived from 22-127 (*lysB-4*, *ben-17*) x 22-135 (*hisA-1*, *sC-6*) on MYG and MYR respectively.

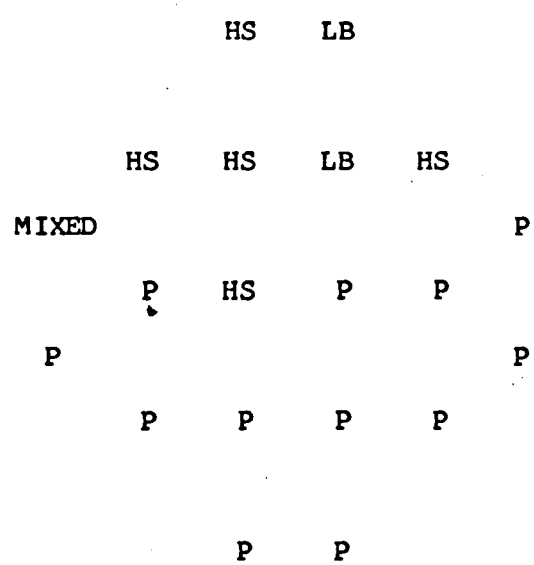
		NUMBER OF COLONIES IN EACH CLASS*				
SOURCE MATERIAL	PROTOTROPH NUMBER	*his*$^-$ *s*$^-$	*lys*$^-$ *ben*^{R}	PROTOTROPHS	OTHER	TOTAL
Spores + hyphal fragments	1	19	7	1	0	27
	2	6	0	66	1	73
	3	14	24	2	0	40
Protoplasts	4	32	16	4	8	60
	5	0	1	59	0	60
	6	22	27	6	5	60

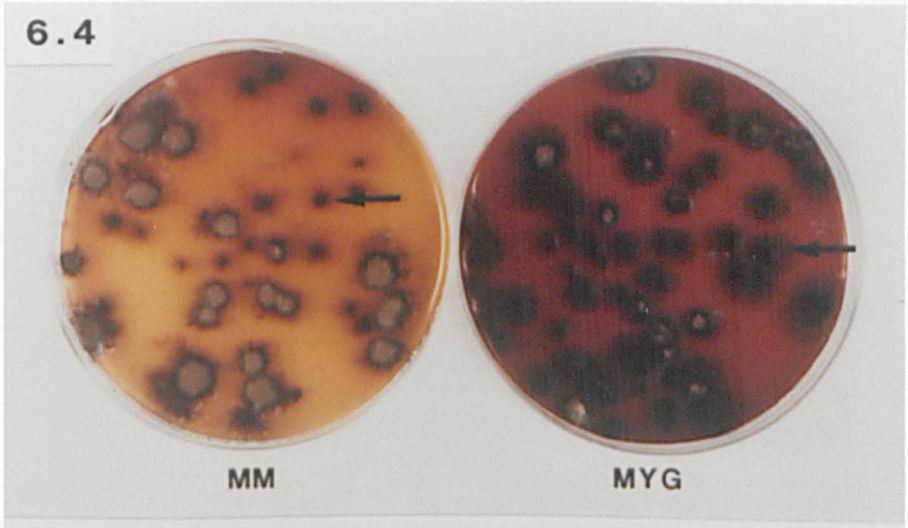
* *his*$^-$ = histidine-requiring; *s*$^-$ = cysteine-requiring; *lys*$^-$ = lysine-requiring; *ben*^{R} = carbendazim-resistant.

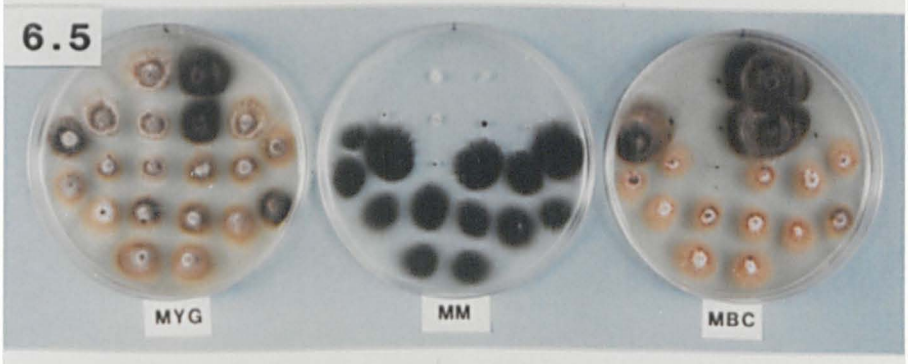
The *ben-17* mutation carried by 22-127, in addition to conferring a high level of resistance to carbendazim, is associated with increased sensitivity to the phenylcarbamate, MDPC. In contrast to the parent strains the prototrophic colonies produced from 22-127 and 22-135 (ben$^+$) were resistant to both 10 μM carbendazim and 50 μM MDPC, when tested separately. However, the rate of growth was less than the resistant parent on the same concentration of the fungicide.

Figure 6.4 Colonies derived from spores and hyphal fragments from the cross of 22-137 (*hisB-2*, *ben-17*) x 22-184 (*con-2*) growing on complete (MYG) and minimal (MM) media. Colonies characteristic of the *con-2* parent are indicated by arrows.

Figure 6.5 Stable prototrophic (P) colonies from the orthodox cross of 22-127 (*lysB-4*, *ben-17*; LB) x 22-135 (*hisA-1*, *sC-6*; HS) and the parental strains growing on diagnostic media. Colonies were arranged as follows:




6.4
MM
MYG


6.5
MYG
MM
MBC

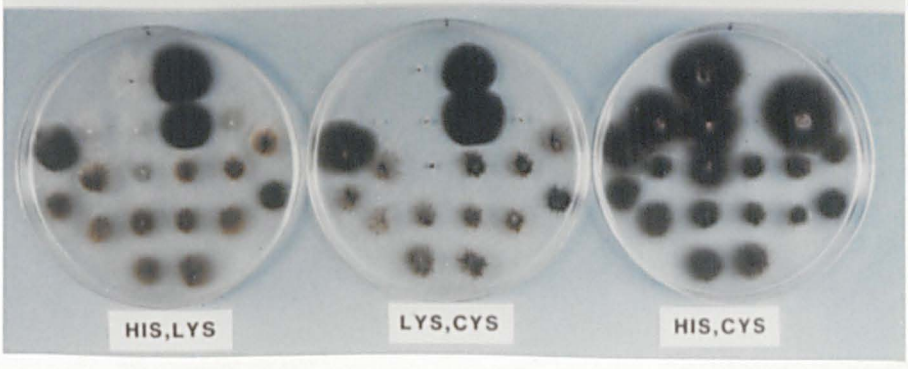

HIS,LYS
LYS,CYS
HIS,CYS

Table 6.5 Stability of prototrophic colonies: Proportion of protoplasts, isolated from prototrophic products of two crosses, producing colonies on complete (MYR) and minimal (MMR) regeneration medium. Each figure represents the mean regeneration frequency of five replicates ± standard error.

PARENTAL STRAINS	PROTOTROPH NUMBER	REGENERATION FREQUENCY (%) MYR	MMR	FREQUENCY OF PROTOTROPHIC REGENERANTS
22-127 x 22-133	3	8.3 ± 1.9	1.0 ± 0.4	12.0
	4	23.6 ± 2.3	8.5 ± 0.8	36.0
	5	20.6 ± 0.8	2.5 ± 0.4	12.1
22-127 x 22-135	4	11.8 ± 2.1	5.5 ± 0.9	46.6
	5	18.5 ± 1.5	16.4 ± 2.1	88.6
	6	17.1 ± 1.6	2.0 ± 1.3	11.7

6.3.1.3 DNA Estimations

Estimation of the DNA content of protoplasts indicated an elevated average DNA content for the nuclei of the prototrophic colonies compared with their parental strains (Table 6.6). While the number of nucleate protoplasts in the samples was relatively small, and the assay consequently at the lower limit for accurate DNA estimation, a repeat experiment confirmed the increased DNA content of the prototrophs.

Table 6.6 Estimated DNA content of protoplasts isolated from 22-127 (*lysB-4*, *ben-17*), 22-135 (*hisA-1*, *sC-6*) and two stable prototrophs (11-13 and 11-17) produced by hyphal anastomosis of these strains.

STRAIN	NUMBER OF PROTOPLASTS IN SAMPLE	PROPORTION OF NUCLEATE PROTOPLASTS	DNA ESTIMATE ($\mu g\ ml^{-1}$)	ESTIMATED DNA CONTENT (μg/nucleus)
22-127	3.91×10^6	47.1%	4.17	2.26×10^{-6}
22-135	7.35×10^6	50.3%	5.17	1.40×10^{-6}
11-13	2.79×10^6	37.0%	4.67	4.52×10^{-6}
11-17	3.97×10^6	44.7%	5.33	3.01×10^{-6}

6.3.1.4 Induced Segregation and Isolation of Recombinants

Twelve of the stable prototrophs from cross 22-127 x 22-135 and the parent strains were point inoculated onto MYG containing 100 µM carbendazim, 50 µM MDPC, 100 $\mu g\ ml^{-1}$ fluorouracil or 200 $\mu g\ ml^{-1}$ fluorouracil, in an attempt to induce segregation. Four replicates of each colony were made on each of the media. Colonies were also inoculated onto unsupplemented MYG.

All colonies grew on these media but no sectors were observed after 21 days incubation. Consequently mycelium from each colony was transferred to TWA, containing all the nutritional supplements required by the parental strains, to allow sporulation. After 2 months, spores from these cultures were plated onto MYG and the resulting colonies classified on the basis of morphology and pigmentation.

Regardless of previous treatment parental strains gave rise only to colonies with the identical phenotype. Conidia from

prototrophs, previously grown on MYG, produced colonies with a range of pigmentation types. Forty-six of the forty-eight independent cultures produced colonies that were variously dark grey-brown, brown-yellow or yellow-white, while the remaining two, in addition to producing colonies of these types, gave a small number (1 to 2 per plate) of slow-growing, unstable, white colonies.

A sample of fast-growing colonies of each pigmentation type was characterised for markers (Table 6.7). Most of the seventy-five colonies tested, from twenty-four separate cultures, were prototrophic and resistant to both fungicides. Fifteen prototrophic colonies, representing twelve separate cultures, were resistant to MDPC and sensitive to carbendazim, while one showed the opposite fungicide response. Six colonies, all of separate origin, expressed one of the parental nutritional requirements. Four required lysine, of which three were resistant to both fungicides, while one was sensitive to carbendazim and resistant to MDPC. The remaining two colonies were histidine auxotrophs, one being doubly resistant, the other resistant only to MDPC.

The colonies derived from prototrophs exposed to 100 µM carbendazim showed a similar range of pigmentation types to those from MYG. However, thirty of the forty-seven separate cultures also yielded slow-growing, unstable, white colonies. A sample of one hundred and nine fast-growing, colonies, from twenty-five separate plates, tested for parental markers showed that most (106) were prototrophic, while three, all originating from the

same plate had a requirement for histidine (Table 6.7). All colonies from prototrophs grown on carbendazim were carbendazim-resistant and sensitive to MDPC.

Table 6.7 Phenotypes of fast-growing colonies derived from spores of prototrophs previously grown on MYG containing carbendazim (MBC), MDPC or 5-fluorouracil (FU). Stable prototrophs, produced by hyphal anastomosis between 22-127 (lysB-4, ben-17) and 22-135 (hisA-1, sC-6), were cultured on MYG containing the compounds for 21 days, prior to transfer to TWA + supplements for spore production. Numbers in parentheses denote the number of independent cultures from which colonies were tested.

PRE-TREATMENT MEDIUM	FREQUENCY OF UNSTABLE COLONIES**	NUMBER OF COLONIES WITH EACH PHENOTYPE*											
		P			H			L		S	HS	TOTAL	
		++	+-	-+	++	+-	-+	++	-+	++	-+		
MYG	4/48	53	15	1	1	0	1	3	1	0	0	75	(24)
10µM MBC	30/47	0	106	0	0	3	0	0	0	0	0	109	(25)
50µM MDPC	2/46	0	0	46	0	0	0	0	0	0	5	51	(19)
100µg mg^{-1} FU	24/26	NT			NT			NT		NT	NT	NT	
200µg ml^{-1} FU	37/43	113	28	1	1	0	0	5	0	1	1	157	(40)

* P = prototrophic; H = histidine-requiring; L = lysine-requiring; S = cysteine-requiring; ++ = resistant to both carbendazim (MBC) and MDPC; +- = resistant to carbendazim, sensitive to MDPC; -+ = sensitive to carbendazim, resistant to MDPC; NT = colonies not tested for parental markers.

** Proportion of plates containing slow-growing, unstable colonies.

Conversly all the fast-growing colonies tested from the MDPC-exposed prototrophs were MDPC-resistant and sensitive to carbendazim (Table 6.7). Most of these colonies were prototrophic, the few auxotrophs obtained being identical to the his⁻ s⁻ parent (22-135).

A greater range of colony types was obtained from spores of prototrophs exposed to fluorouracil. Most of the cultures produced the slow-growing, unstable, white colony type, a few almost exclusively. The remaining fast-growing colonies were variously coloured black or grey-brown to yellow or white. Two of these plates are shown in Figures 6.6 and 6.7, one consisting predominantly of slow, unstable colonies, the other containing a number of colony types.

A sample of one hundred and fifty-seven fast-growing colonies, from forty separate plates, were characterised further (Table 6.7). As was found for the other treatments, the majority of these colonies were prototrophic and resistant to both fungicides. Only seven colonies had nutritional requirements, of which all but the his⁻ s⁻ colonies were doubly resistant.

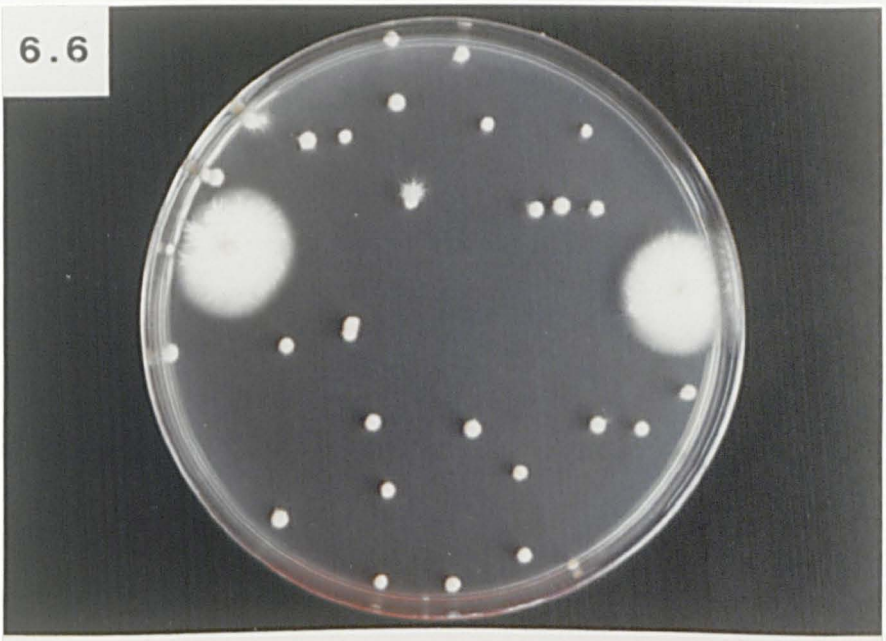
6.3.1.5 Purification of Slow-growing, Unstable Colonies

Thirty-two of the slow-growing, unstable colonies, from eleven separate cultures, obtained following fluorouracil exposure, were 'purified' by plating a suspension of macerated mycelium onto fresh MYG. A great variety of colony types were recovered, differing in growth rate, pigmentation and stability (Fig. 6.8). Samples of colonies taken from these purification plates were tested for auxotrophic and resistance markers.

Figure 6.6 Slow-growing, unstable colonies on MYG derived from spores and hyphal fragments of the stable prototroph Number 389 (22-127; lysB-4, ben-17 x 22-135; hisA-1, sC-6). Conidia were produced on TWA plus supplements from mycelium previously grown on MYG containing 200 μg m^{-1} 5-fluorouracil. Plate photographed after 14 days incubation.

Figure 6.7 Range of colony types obtained on MYG from spores and hyphal fragments of the stable prototroph Number 395. Conidia were produced on TWA plus supplements from mycelium previously grown on MYG containing 200 μg m^{-1} 5-fluorouracil. Plate photographed after 14 days incubation. Slow-growing colonies near top of plate indicated by an arrow.

6.6



6.7

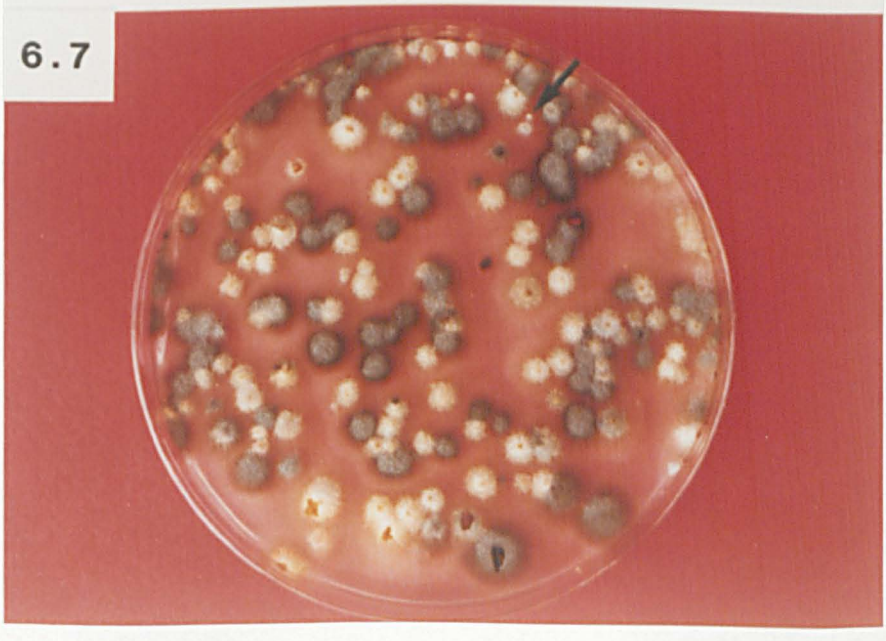
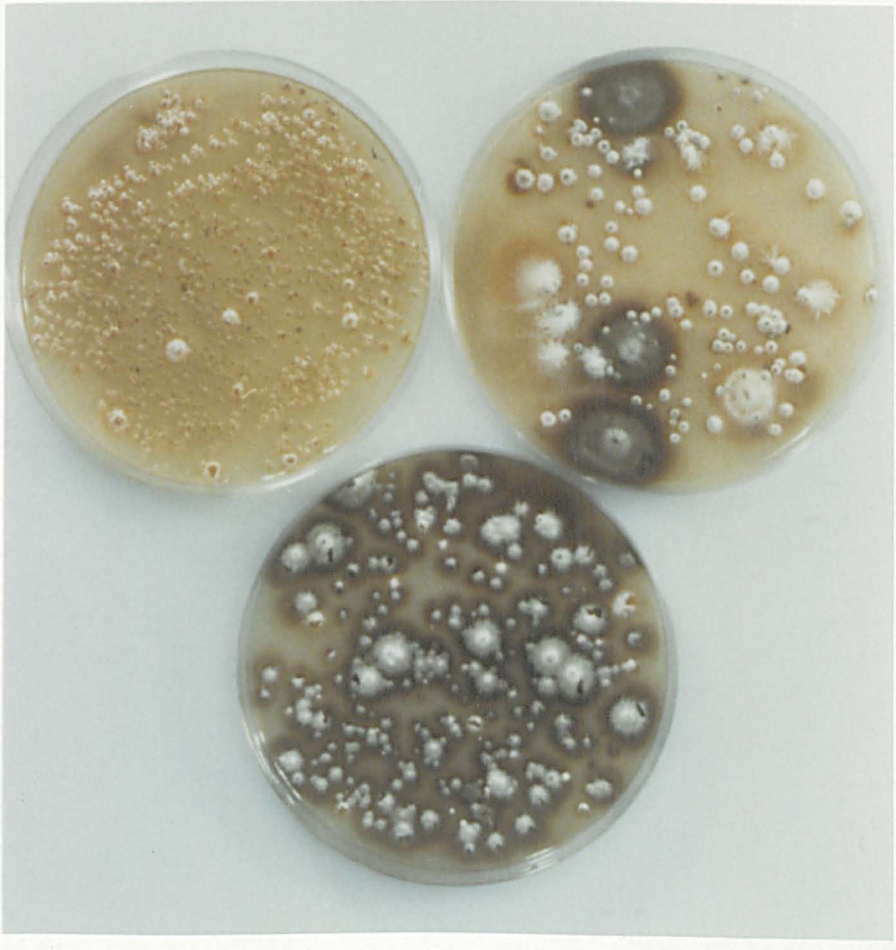


Figure 6.8 Colonies obtained by purification of three separate slow-growing, unstable colonies derived from stable prototroph Number 393 after exposure to 5-fluorouracil. Plates photographed after 18 days growth.

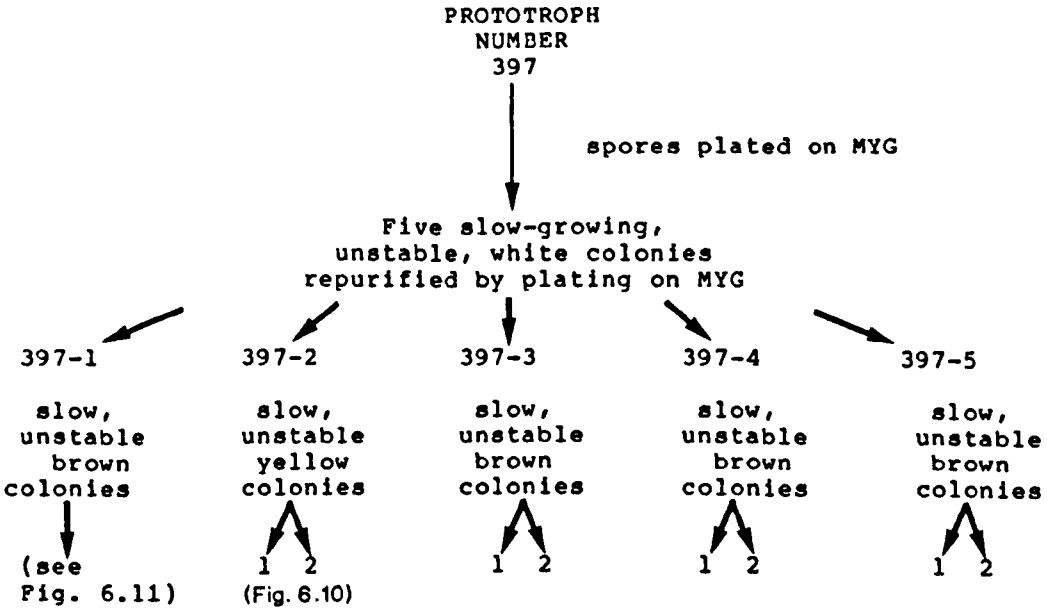


Although many of the colonies proved to be prototrophic, with a range of resistance phenotypes, several plates yielded colonies with one or more nutritional requirements. Many of the colonies were repurified and retested on the diagnostic media. Since the genealogy of these colonies becomes increasingly complex, only a few will be described in more detail as examples.

Of the forty-nine colonies obtained by plating spores of one prototroph (No. 397), previously exposed to 200 μg ml^{-1} fluorouracil, twelve were slow-growing and white. Five of these small colonies (397-1,2,3,4,5) were purified by plating on MYG (Fig. 6.9). One (397-2) produced many yellow-white, unstable colonies (Fig. 6.10). Twenty-three of these colonies were tested for parental markers, all were prototrophic and all but three resistant to both fungicides. Nine of the yellow-white colonies were repurified by plating on MYG. Most of the resulting colonies characterised were prototrophic and doubly resistant. A few were carbendazim-sensitive and one prototrophic colony was sensitive to both carbendazim and MDPC. A single reduced sulphur-requiring colony was recovered, which was sensitive to carbendazim and resistant to MDPC.

Three of the other colonies purified on MYG (379-3,4,5) produced slow-growing, unstable, brown, densely-sporing colonies (Fig. 6.11). Characterisation of samples of these colonies and their sectors revealed that most were prototrophic and resistant to both fungicides (Fig. 6.9). A proportion of the colonies derived from 397-3 were auxotrophic, two requiring cysteine and one lysine. All three were resistant to MDPC and sensitive to

Figure 6.9 Re-purification of five slow-growing, unstable colonies derived from prototroph number 397. Colonies obtained on MYG were either directly transferred to MYG master plates for phenotype characterisation or re-purified by plating on MYG and the resulting colonies tested for parental marker segregation.



1 MYG MASTER PLATES ⟶ PHENOTYPE CHARACTERIZATION

PHENOTYPES*			Number of colonies with each phenotype			
	MBC	MDPC	397-2	397-3	397-4	397-5
P	+	+	20	12	13	14
P	+	-	0	0	0	0
P	-	+	3	0	2	1
S	-	+	0	2	0	0
L	-	+	0	1	0	0
TOTAL			23	15	15	15

2 REPURIFICATION ON MYG ⟶ MASTER PLATES ⟶ PHENOTYPE CHARACTERIZATION

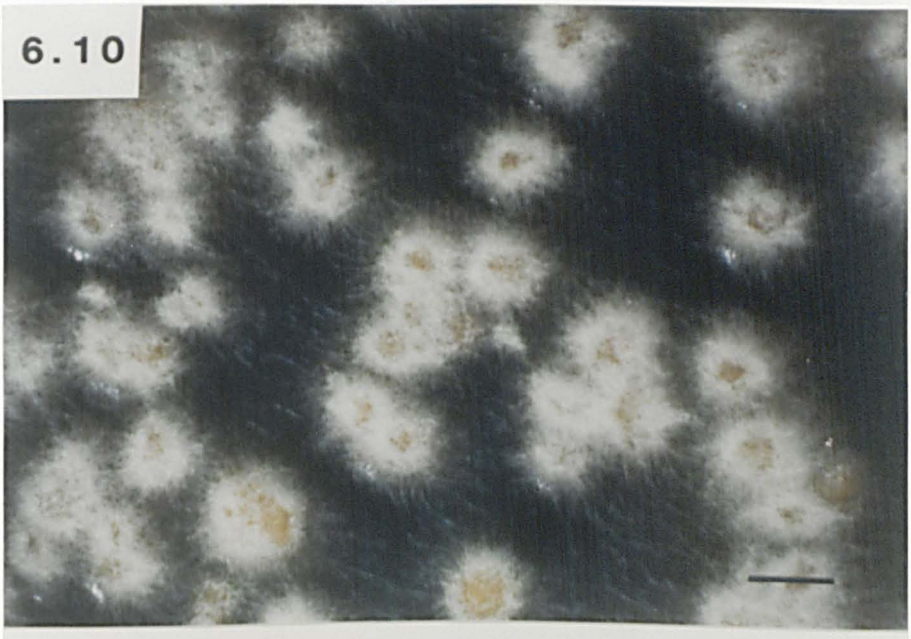
PHENOTYPES*			Number of colonies with each phenotype			
	MBC	MDPC	397-2	397-3	397-4	397-5
P	+	+	37	33	29	27
P	+	-	0	0	1	1
P	-	+	5	3	3	1
P	-	-	1	0	0	0
S	-	+	1	6	0	0
L	+	-	0	0	0	1
L	-	+	0	14	0	0
LS	-	+	0	3	0	1
TOTAL			44 (9)**	56 (15)	33 (8)	31 (7)

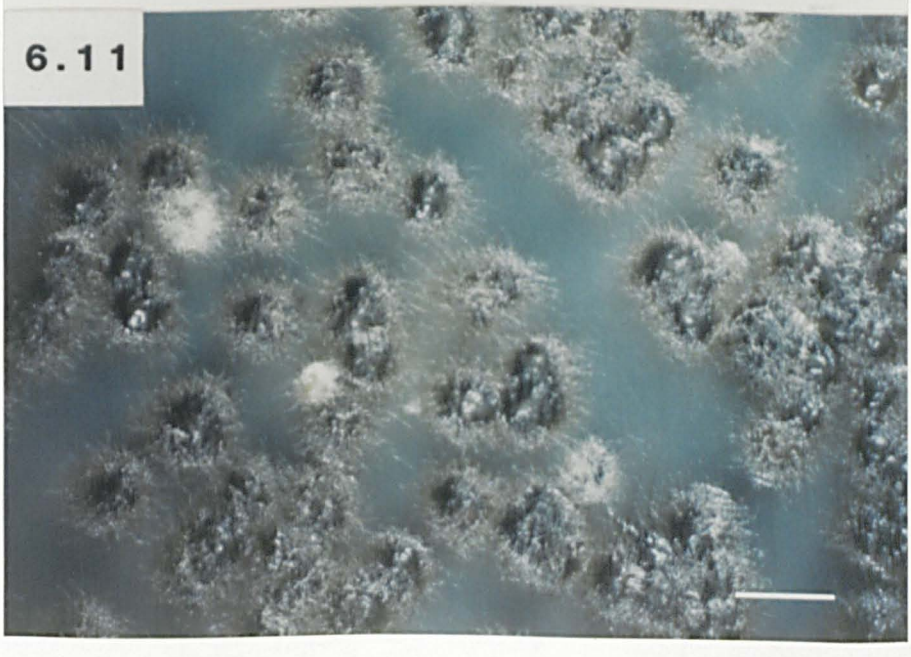
* P = Prototrophic; S = s^-; L = $\underline{lys}^-$; + = resistant to carbendazim (MBC) or MDPC; - = sensitive to carbendazim (MBC) or MDPC.

** Figures in parentheses denote number of separate cultures from which colonies were tested.

Figure 6.10 Yellow-white, unstable colonies obtained by purification of the slow-growing, unstable colony Number 397-2 on MYG. Bar represents 2 mm.

Figure 6.11 Dark, unstable, sporing colonies obtained by purification of the slow-growing, unstable colony Number 397-1 on MYG. Bar represents 2 mm.


6.10


6.11

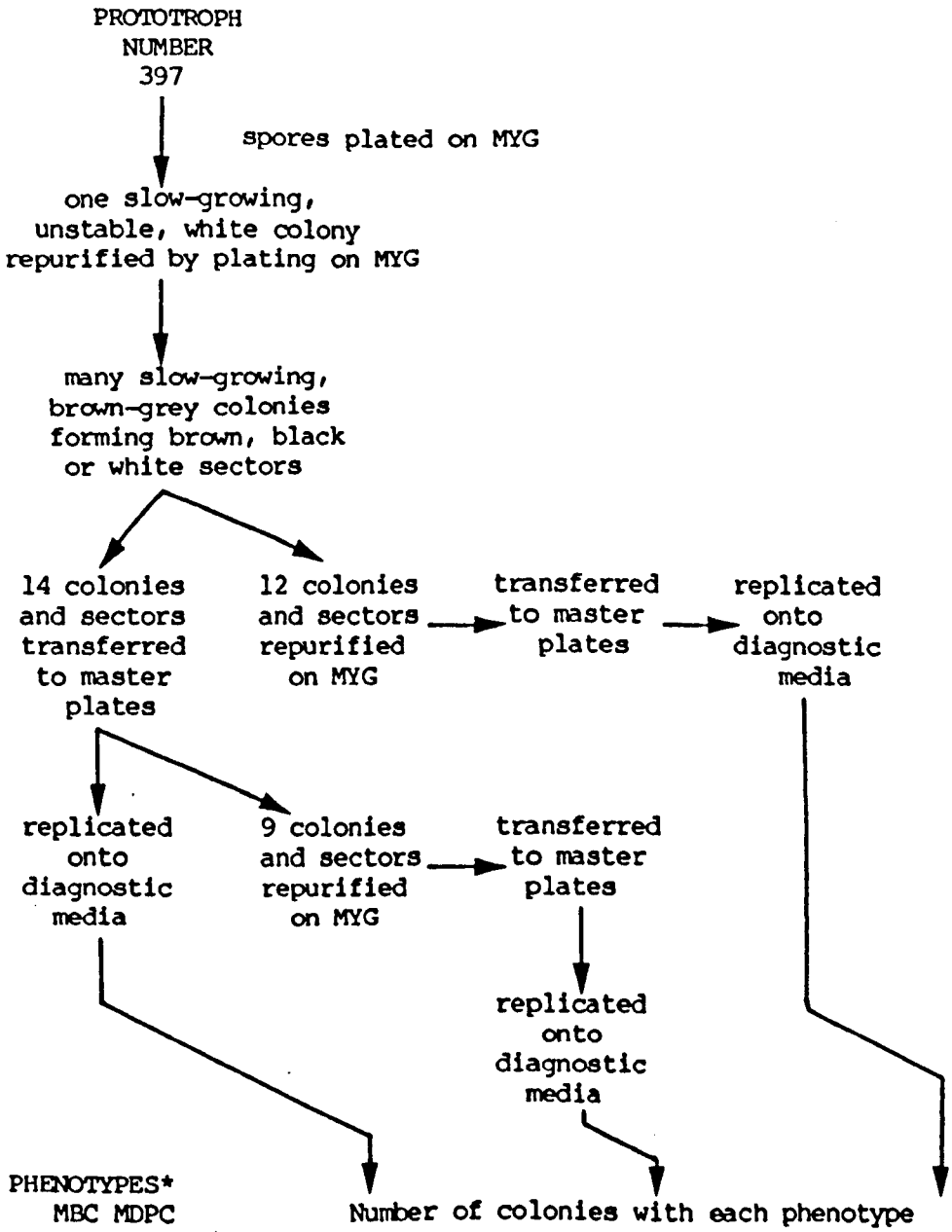
carbendazim. A greater range of auxotrophic phenotypes was obtained after repurification of some of the colonies on MYG (Fig. 6.9).

This feature of increasing numbers of recombinant auxotrophic types being recovered after repeated purification is best illustrated by the derivatives from 397-1 (Fig. 6.12). The colonies produced on MYG from plating hyphal fragments of this colony were slow-growing, brown-grey and sectored readily to give white, brown or black areas. Fourteen of these colonies and their sectors were transferred to MYG master plates for characterisation and, at the same time, twelve were repurified by plating on MYG. A number of the colonies on the master plates formed new sectors prior to transfer to the diagnostic plates. These sectors and their colony centres were again repurified on MYG. As can be seen from Figure 6.12, the colonies growing on the original master plates were all prototrophic and most doubly resistant. By contrast, a range of auxotrophic recombinant phenotypes was recovered from the repurified progeny.

Purifications of sectors generally produced fast-growing colonies of the same type, although different sectors gave rise to colonies with very different morphologies. The repurified centres of such sectoring colonies, however, usually produced many slow-growing equally unstable colonies. Some of these purified colonies and sectors are shown in Figure 6.13.

Conidia from a different prototroph (356), plated on MYG yielded only four fast-growing colonies. These were shown to be double auxotrophs, requiring both histidine and cysteine, and carbendazim sensitive. In addition, three very slow-growing,

Figure 6.12 Re-purification of slow-growing, unstable colony, 397-1, derived from prototroph number 397. Colonies and sectors growing on MYG were either directly transferred to MYG master plates or re-purified by plating on MYG. Colonies sectoring on master plates re-purified again before phenotype characterisation.



PHENOTYPES*			Number of colonies with each phenotype		
	MBC	MDPC			
P	+	+	10	7	3
P	+	-	3	8	14
P	-	+	2	7	12
S	+	-	0	0	1
S	-	+	0	6	2
L	+	-	0	4	0
L	-	+	0	0	0
LS	+	-	0	0	1
LS	-	+	0	0	2
TOTAL			15	32	35

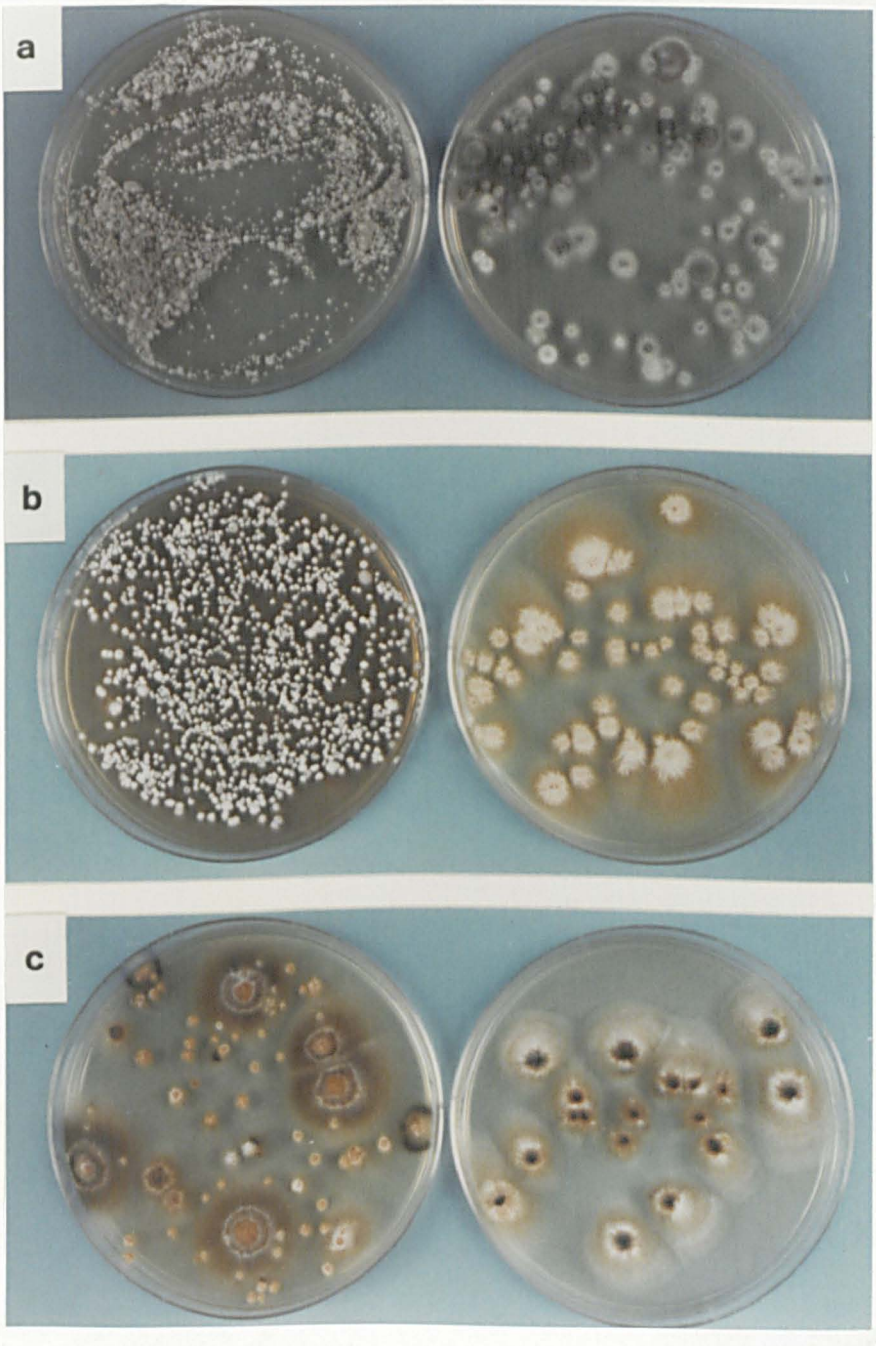
* P = protoptrohic; S = $\underline{s}^-$; L = $\underline{lys}^-$; + = resistant to carbendazim (MBC) or MDPC; - = sensitive to carbendazim (MBC) or MDPC.

Figure 6.13 Re-purification of slow-growing, unstable colonies: colonies producing sectors on MYG were re-purified by plating macerated mycelium on MYG. Mycelium was taken from the colony centres (left) and sectors (right), and plates were incubated for 16 days before photographing.

a re-purification of sectoring colony from 397-1.

b re-purification of sectoring colony from 368-2.

c re-purification of sectoring colony from 397-2.


a
b
c

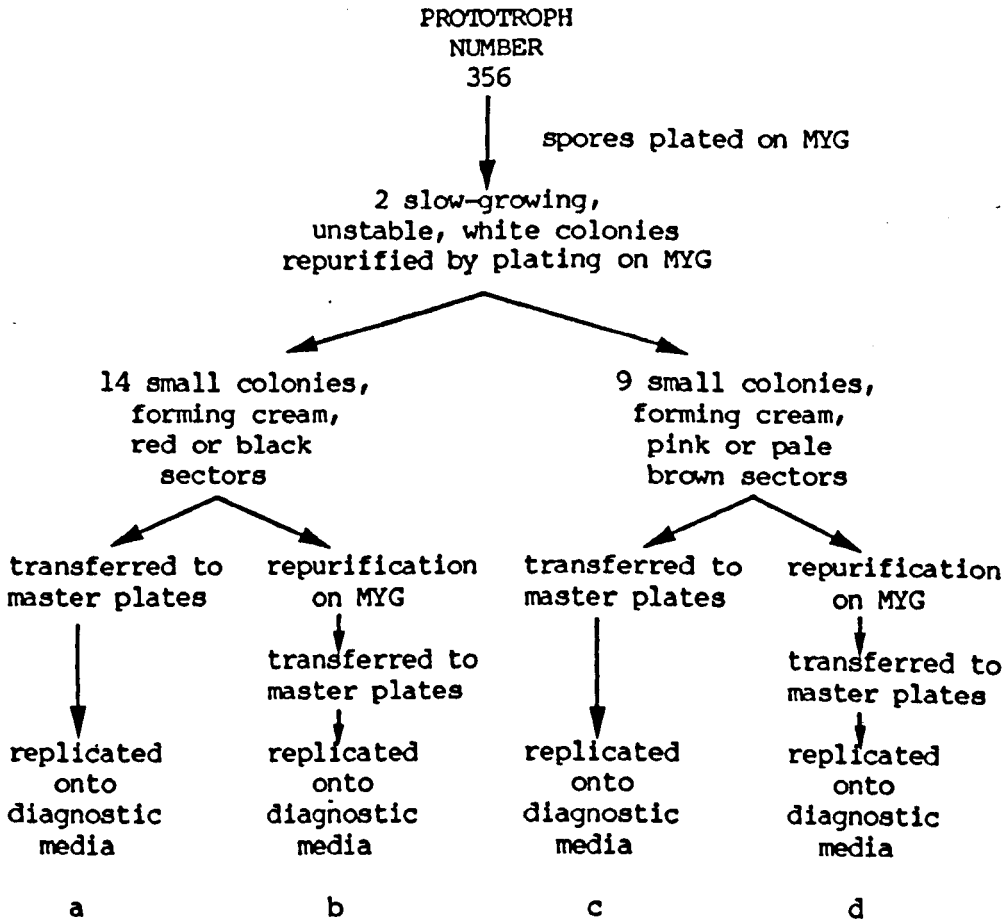
unstable, white colonies were obtained, two of which (356-1,2) were purified by plating on MYG (Fig. 6.14). In both cases a small number of slow-growing, white colonies were produced, which sectored to give cream-white, pink, brown or black regions. Colonies and sectors were transferred to master plates for phenotype characterisation. At the same time mycelium from the same colonies was plated on MYG for repurification.

The range of phenotypes recovered is presented in Figure 6.14. All derivatives of the slow-growing colony 356-1 were auxotrophic with requirement genotypes for either *his*$^-$, *his*$^-$ *s*$^-$ or *his*$^-$ *s*$^-$ *lys*$^-$. After repurification only the double and triple auxotrophs were recovered.

Most of the colonies derived from 356-2 were single auxotrophs requiring histidine. After repurification a small number of colonies was obtained requiring both histidine and cysteine, all from the same purification plate. The fungicide response of the majority of colonies was typical of one or other of the original parents, 22-127 or 22-135. Several, however, were either resistant or sensitive to both compounds.

Segregation of auxotrophic markers is most clearly illustrated by the colony shown in Figure 6.15. This unstable colony, derived from 356-1, produced two morphologically dissimilar sectors on MYG. After purification of both sectors and the colony centre the resulting progeny were tested for parental markers. All the colonies derived from one sector were double auxotrophs (*his*$^-$, *s*$^-$), while those from the other required all three supplements (*his*$^-$, *s*$^-$, *lys*$^-$). Colonies originating from the central region were of either type.

Figure 6.14 Re-purification of two slow-growing, unstable colonies derived from prototroph 356. Colonies and sectors produced on MYG were either directly transferred to MYG master plates for phenotype characterisation or re-purified again by plating on MYG prior to testing for parental marker segregation.



PHENOTYPES*			Number of colonies with each phenotype			
			356-1		356-2	
	MBC	MDPC	a	b	c	d
H	+	+	2	0	6	29
H	+	-	0	0	3	27
H	-	+	0	0	7	31
H	-	-	0	0	0	2
HS	+	+	3	2	0	0
HS	+	-	3	27	0	6
HS	-	+	3	23	0	0
HS	-	-	1	4	0	0
HLS	+	+	1	1	0	0
HLS	+	-	1	23	0	0
HLS	-	+	2	1	0	0
TOTAL			16	81 (8)**	16	95 (11)

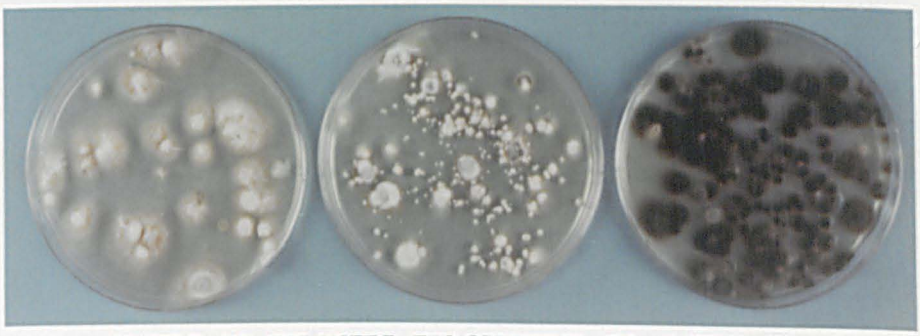
* P = prototrophic; H = his$^-$; S = s$^-$; L = lys$^-$; + = resistant to carbendazim (MBC) or MDPC; - = sensitive to carbendazim (MBC) or MDPC.

** Numbers in parentheses denote number of separate cultures from which colonies were tested.

Figure 6.15 Segregation of auxotrophic markers in a single unstable colony by purification of colony 356-1. Sectors and colony centre were repurified by plating on MYG and tested for parental markers.

REPURIFICATION
OF 356-1

PURIFICATION OF SECTORS AND CENTRE ON MYG



SAMPLE OF COLONIES TRANSFERRED TO MASTER PLATES

REPLICATED ONTO DIAGNOSTIC MEDIA
FOR PHENOTYPE CHARACTERISATION

a b c

PHENOTYPES*		Number of colonies with each phenotype		
MBC	MDPC	a	b	c
HS +	-	0	2	7
HLS +	-	20	2	0
TOTAL		20	4	7

* H = his$^-$; S = s$^-$; L = lys$^-$; + = resistant to carbendazim (MBC) or MDPC; - = sensitive to carbendazim (MBC) or MDPC.

6.3.1.6 Resistance Expression

It was observed that many purified colonies appeared doubly resistant, and a few doubly sensitive, to the two fungicides. Often the growth on one or both fungicides was slow and the colonies formed were small and irregular (Fig. 6.16). To establish whether these were recombinant phenotypes rather than simply derived from heterogeneous colonies a proportion were repurified by plating mycelial fragments on MYG, and the resulting colonies re-tested on the fungicide-containing media. In addition, mycelium from colonies growing on the fungicide-containing plates was directly re-tested for response to both compounds.

In almost all instances, re-purification of colonies, apparently resistant to both fungicides, yielded a proportion which were sensitive to one or other compound. Mycelium taken from the original diagnostic plates and re-tested, usually showed resistance only to the fungicide on which the original was growing (Table 6.8). Some important ex-ceptions were found. Many colonies grew slowly on one or both fungicides, producing colonies similar in morphology to the original unstable, slow-growing forms. Mycelium taken from these colonies and re-tested for resistance to the two compounds, occasionally produced growth on both fungicide plates (Table 6.8; numbers 356-3, 397-6 and 332-1). Similarly colonies initially with slow irregular growth on MYG containing MDPC, and which ultimately sectored to give a faster growing colony margin, were shown to be heterogeneous with respect to fungicide response (Table 6.8; numbers 332-1 and 393-1). Mycelium re-tested from the central region of these colonies

Figure 6.16 Purification products of 356-1 growing on diagnostic plates showing segregation of resistance markers. Some colonies (e.g. Second row, far left) grew normally on one fungicide but formed small lumpy colonies on the other, while a few others (e.g. Middle row, second left) appeared sensitive to both fungicides.

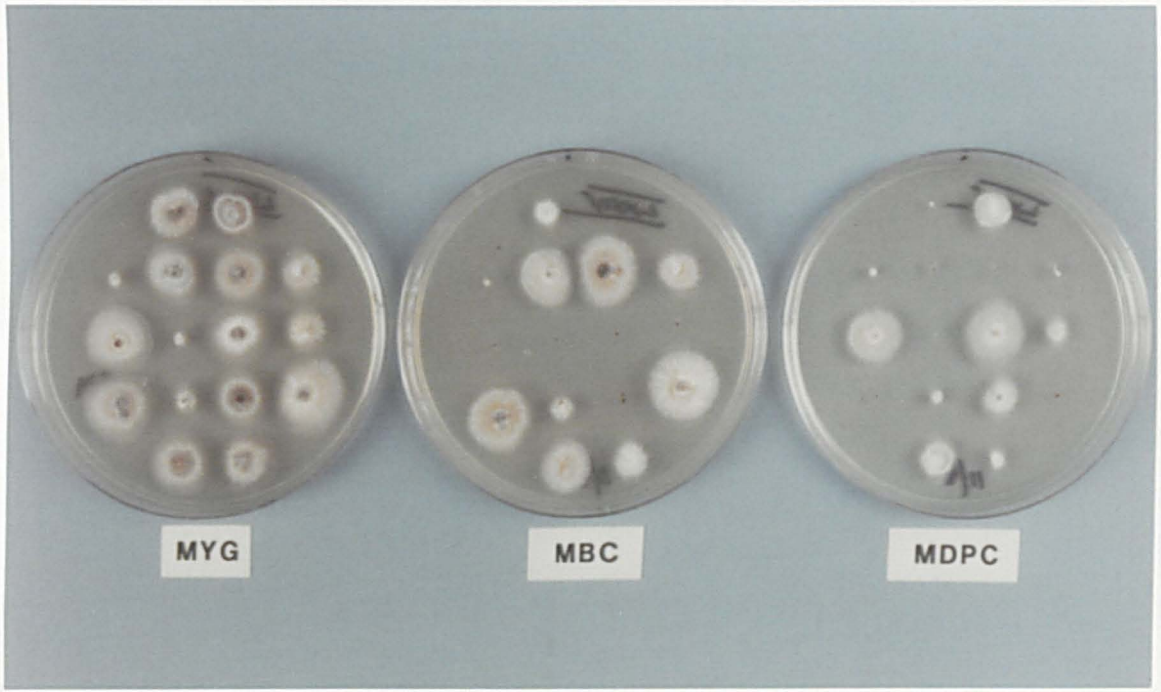

MYG
MBC
MDPC

Table 6.8 Fungicide response of mycelium taken from colonies growing on fungicide-containing plates. All colonies were derived from fluorouracil-exposed prototrophs and had been repurified at least once by plating on MYG prior to the original resistance phenotype assessment

COLONY NUMBER	TEST FUNGICIDE	ORIGINAL FUNGICIDE RESPONSE	FUNGICIDE RESPONSE* ON RETESTING OF MYCELIUM TAKEN FROM: MYG	MBC	MDPC
331-1	0	+	+	+	+
	MBC	+	+	+	-
	MDPC	+	+	-	+
338-1	0	+	+	+	+
	MBC	(+)	+	+	-
	MDPC	+	+	-	+
338-2	0	+	+	+	+
	MBC	(+)	+	+	-
	MDPC	+	+	-	+
338-3	0	+	+		
	MBC	-	+	NT	NT
	MDPC	-	+		
372-1	0	+	+	(+)	+
	MBC	(+)	+	(+)	-
	MDPC	+	+	-	+
372-2	0	+	+		+
	MBC	-	+	NT	(+)
	MDPC	+	+		+
356-3	0	+	+	+	+
	MBC	+	(+)	+	(+)
	MDPC	(+)	+	-	+
397-6	0	+	+		(+)
	MBC	-	(+)	NT	(+)
	MDPC	(+)	+		(+)

Table 6.8 continued

COLONY NUMBER	TEST FUNGICIDE	ORIGINAL FUNGICIDE RESPONSE	FUNGICIDE RESPONSE* ON RETESTING OF MYCELIUM TAKEN FROM: MYG	MBC	MDPC	
					centre	edge
332-1	0	+	+	+	+	+
	MBC	(+)	+	+	(+)	-
	MDPC	+***	+	-	+	+
393-1	0	+	+	+	+	+
	MBC	+**	+	+	(+)	-
	MDPC	+***	+	-	+	+

* + = resistant; - = sensitive; (+) = resistant but colony small and lumpy; NT = not tested.
** Colony margin irregular.
*** Colony slow-growing, with white centre and darker margin.

produced growth on either compound, while that from the colony margin grew only on MDPC.

A small number of colonies were apparently sensitive to both carbendazim and MDPC. Mycelium taken from these colonies, growing on fungicide-free medium, however, invariably showed resistance to one or both compounds when re-tested (Table 6.8; number 338-3). Furthermore, resistant types were always recovered after re-purification of such colonies.

6.3.2 Protoplast Fusion

An alternative method to hyphal anastomosis for the recovery of heterokaryons involves the fusion of isolated protoplasts of the parental strains. This technique has been used in several species of fungi to overcome vegetative incompatibility systems which preclude orthodox mating procedures (Dales & Croft, 1977; Typas, 1983; Zhemchuzhina et al, 1985).

Furthermore the technique makes possible various novel selection systems, such as the use of dead donors, which avoid the need for auxotrophic markers in both parental strains.

To establish whether viable heterokaryons could be produced by protoplast fusion in P. herpotrichoides, protoplasts were prepared from two compatible strains with complementary auxotrophic markers (22-133, hisA-1, metB-2; 22-139, argA-1, red-1). The protoplasts were mixed, induced to fuse using PEG in the presence of Ca^{++} ions, and plated onto MMR and CMR. Control platings included un-fused mixtures of protoplasts and PEG treated and un-treated protoplasts of the individual parents.

Rapidly-growing, prototrophic colonies appeared on the plates inoculated with fused mixtures of protoplasts after 14 to 21 days (Fig. 6.17). The frequency of recovery of these "fusion products", together with the regeneration rates of the parental strainsare given in Table 6.9.

Ninety dark pigmented fusion products were transferred to fresh MM. After 9 days the growth and appearance of the colonies was visually assessed. All of the sub-cultures produced prototrophic mycelium on MM. The colonies were dark brown with a less heavily pigmented margin (Fig. 6.18).

A background growth of hyaline slow growing mycelium was discernible on all plates inoculated with protoplasts of 22-139 (Fig. 6.17). One hundred and twenty of these colonies were transferred to MM. In each case slow, non-pigmented growth, characteristic of strain 22-139 was obtained. Protoplasts of the other strain in the cross, 22-133, only produced colonies on CMR

Table 6.9 Protoplast regeneration and fusion frequency for strains 22-133 and 22-139. Colony numbers counted after 16 days incubation at 19°C. Each figure represents the mean of five replicate platings on complete (MYR) or minimal (MMR) regeneration medium ± standard error.

STRAIN COMBINATION	PEG TREATMENT	REGENERATION FREQUENCY (%) COMPLETE MEDIUM	MINIMAL MEDIUM
22-133	UNTREATED	83.5 ± 3.3	0
	PEG-TREATED	1.9 ± 0.2	-
22-139	UNTREATED	82.4 ± 12.7	38.5 ± 4.3*
	PEG-TREATED	2.4 ± 0.2	-
22-133 x 22-139	UNTREATED	-	0.04 ± 0.01
	PEG-TREATED	2.1 ± 0.1	0.12 ± 0.02
FUSION FREQUENCY = 5.7%			

* Colonies small, slow-growing and hyaline

or MMR supplemented with histidine and methionine. All of the 60 regenerant colonies of this strain, tested on MM, were auxotrophic. A small number of prototrophic colonies were obtained from mixed platings of protoplasts not treated with PEG. These colonies presumably resulted from hyphal anastomosis between the two parents from protoplasts regenerating in close proximity.

Demonstration of the heterokaryotic nature of the fusion products requires the re-isolation of both parental typesfrom these colonies. Two methods, isolation of single hyphal tips and plating of conidia and hyphal fragments taken from the surface of

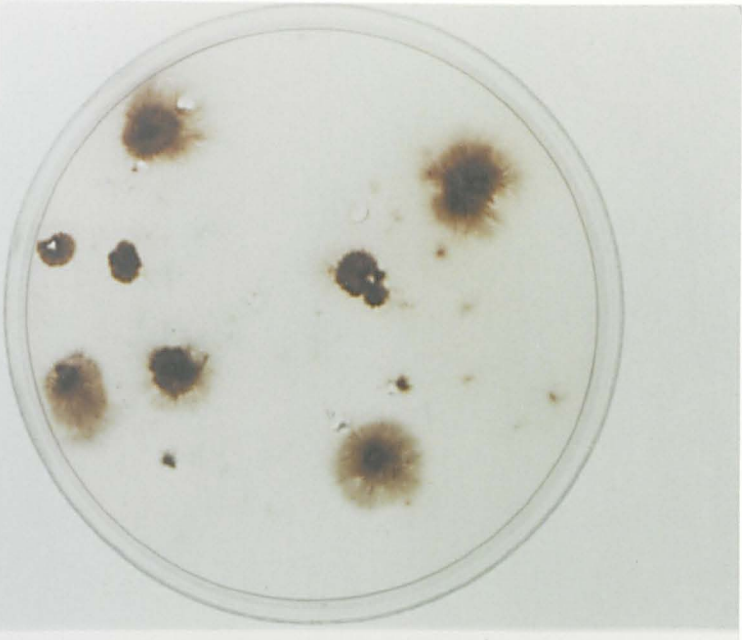
the colonies, were used to recover the parental strains from the prototrophs. Sixteen separate hyphal tips were taken from the margins of five different prototrophic colonies, growing on MM and transferred to MM. In each case slow-growing hyaline colonies resulted, which produced normal colonies when inoculated onto MM supplemented with arginine.

Suspensions of hyphal fragments and conidia from the surface of eight prototrophic colonies were plated onto MYG and MM. A sample of the colonies produced on MYG were tested for parental auxotrophic markers (Table 6.10). Both parental types were recovered from seven of the platings, although the majority of the auxotrophic colonies were of the 22-139 parental type. In addition, prototrophic colonies were obtained. Many of these were visibly heterogeneous, presumably originating from heterokaryotic mycelium. However several apparently stable, prototrophic colonies were recovered; these were unlike either parent strain in appearance, having grey aerial mycelium and, when veiwed from below, a bright orange colouration. Unfortunately, insufficient time was available to allow further analysis of these colonies.

Figure 6.17 Prototrophic colonies, produced by protoplast fusion between 22-133 (hisA-1, metB-2) and 22-139 (argA-1, red-1), growing on osmotically stabilized minimal medium. Plate photographed 28 days after inoculation.

Figure 7.3 Vegetative incompatibility in P. herpotrichoides. Dark bands of heterokaryotic growth produced at the junction of unrelated compatible strains, carrying complementary auxotrophic markers, are indicated by arrows.

6.17



7.3

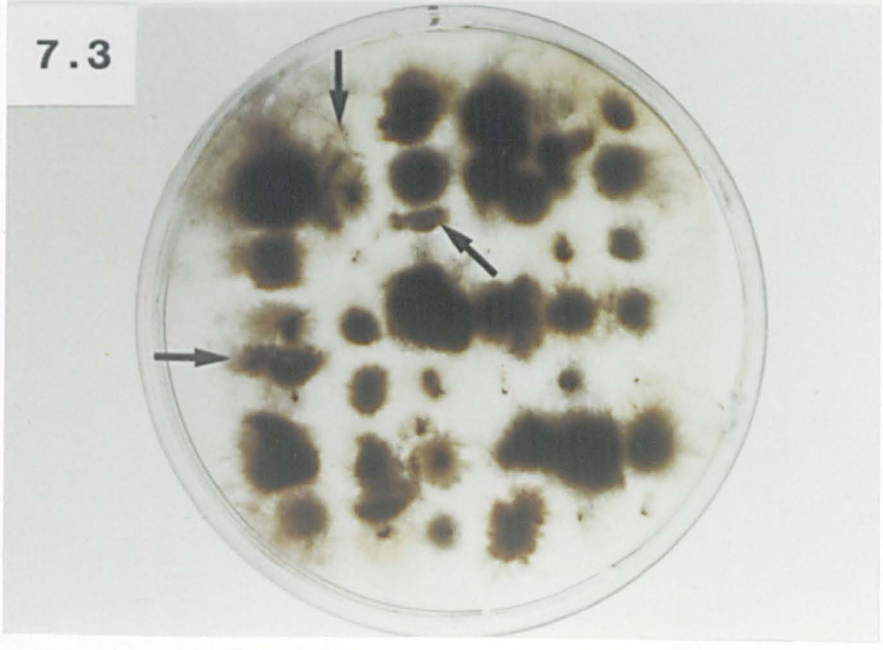


Figure 6.18 Protoplast fusion in P. herpotrichoides: parental strains on complete (top) and minimal (middle) medium. Left: 22-133 (hisA-1, metB-2); right: 22-139 (argA-1, red-1). Bottom row: fusion products on minimal medium.


his-1,met-2
arg-1,red-1
CM
MM
his-1,met-2 x arg-1,red-1
MM

Table 6.10 Phenotypes of colonies recovered after plating spores and hyphal fragments from eight prototrophs, obtained as fusion products of 22-133 (*hisA-1*, *metB-2*) and 22-139 (*argA-1*, *red-1*).

PROTOTROPH NUMBER	NUMBER OF COLONIES WITH EACH PHENOTYPE				
	arg⁻	*his*⁻*met*⁻	PROTOTROPHS	OTHER	TOTAL
1	14	1	0	0	15
2	13	0	3*	0	16
3	14	2	0	0	16
4	10	5	0	0	15
5	7	2	2	4	15
6	4	1	7	4	16
7	5	3	7	1	16
8	7	1	4*	1	12

*These prototrophic colonies included some which were apparently homogeneous and pigmented orange-grey.

6.4 DISCUSSION

A parasexual cycle, amenable to manipulation in the laboratory, clearly does occur in *P. herpotrichoides*. This demonstration confirms the report of Davies and Jones (1970a) of the isolation of a stable diploid "hybrid" produced by hyphal anastomosis beyween two isolates and the recovery of the parental types after treatment with FPA.

The first stage in the cycle, heterokaryon formation, can be induced either by hyphal anastomosis or via protoplast fusion. The heterokaryotic stage does not seem to have a stable, balanced

organisation like heterokaryons of Aspergillus spp., but appears to be similar to that described first for Verticillium dahliae (Puhalla & Mayfield, 1974). Most of the vegetative cells of P. herpotrichoides are uninucleate, the heterokaryotic phase is presumably restricted, as with V. dahliae, to individual anastomosed cells. Such anastomosis bridges are readily formed between neighbouring hyphae in Pseudocercosporella. This view of heterokaryon structure is supported by the observation that hyphal tips, isolated from heterokaryotic mycelium, give rise only to parental type colonies.

Three lines of evidence suggest that the stable prototrophic colonies derived from strains 22-127 and 22-135 are diploids:
1) stable transmission via single spores and hyphal fragments and, more particularly, via uninucleate protoplasts
2) colorimetric estimations indicate an increased DNA content of the nuclei of prototrophs, compared with the parental strains
3) progeny with new combinations of the parental markers were isolated from these colonies.

Additional criteria for identification of diploid colonies in fungi include differences in spore size and in the ratio of cell volume to nuclear number of apical cells (Pontecorvo, 1956; Clutterbuck, 1969). Spore formation by the stable prototrophs was poor, the conidia produced, however, did not appear significantly larger than those formed by the parental isolates and contained a comparable number of cell compartments. Liquid culture-grown mycelium of both prototrophs and parental strains was examined for differences in apical cell size and nuclear content. While great variation in cell length was observed in all preparations,

most cells contained only a single nucleus.

Recombinant progeny were obtained after exposure of stable prototrophs to 5-fluorouracil. This agent is primarily a promoter of mitotic crossing-over. Consequently many of the segregants obtained may have been the result of mitotic cross-over events, followed by segregation of the recombinant chromatids, as described in the introduction to this chapter. Since mitotic recombination is unlikely to alter the euploid chromosome complement, segregant colonies produced by this process will appear identical to the original diploid, provided no mutants affecting colony morphology are located on the recombinant chromosome arm. Several of the fast-growing colonies recovered from stable prototrophs differed from the original colonies either in fungicide response or nutritional requirement, while resembling the original "diploids" in colony appearance. It is possible that these segregant colonies were mitotic recombinant types, retaining the diploid chromosome complement. Similarly colonies from prototrophs grown on high levels of carbendazim or MDPC, while retaining the overall appearance of the original colonies, were all segregant for fungicide response. Prolonged exposure to the inhibitor presumably selected for nuclei homozygous for the appropriate resistance allele.

However, the morphology and behaviour of the unstable, slow-growing colonies obtained from many of the cultures, including some not treated with fluorouracil, is strongly suggestive of the aneuploid colonies in A. nidulans (Upshall, 1971) and the unstable heterozygotes in C. acremonium (Hamlyn, 1982; Birkett &

Hamlyn, 1985).

Aneuploid nuclei are produced as a result of non-disjuction of sister chromatids during nuclear division. If this process occurs in a diploid nucleus one of the daughter nuclei will be trisomic for the non-disjunctional chromosome (2n+1), while the other will be monosomic for that chromosome (2n-1). Hyperdiploid nuclei may lose the additional chromosome by a second non-disjunctional event, regenerating the balanced diploid state. Depending on which of the three homologues is lost, the regenerated diploid may be homozygous or heterozygous for the segregating chromosome. Hypodiploid nuclei can generate haploid progeny by repeated non-disjunction, or haploidization. Furthermore, a high frequency of mitotic recombination, such as would be induced by treatment with fluorouracil, may also have resulted in the segregant progeny obtained. In which case the recombinant colonies would necessarily be diploid.

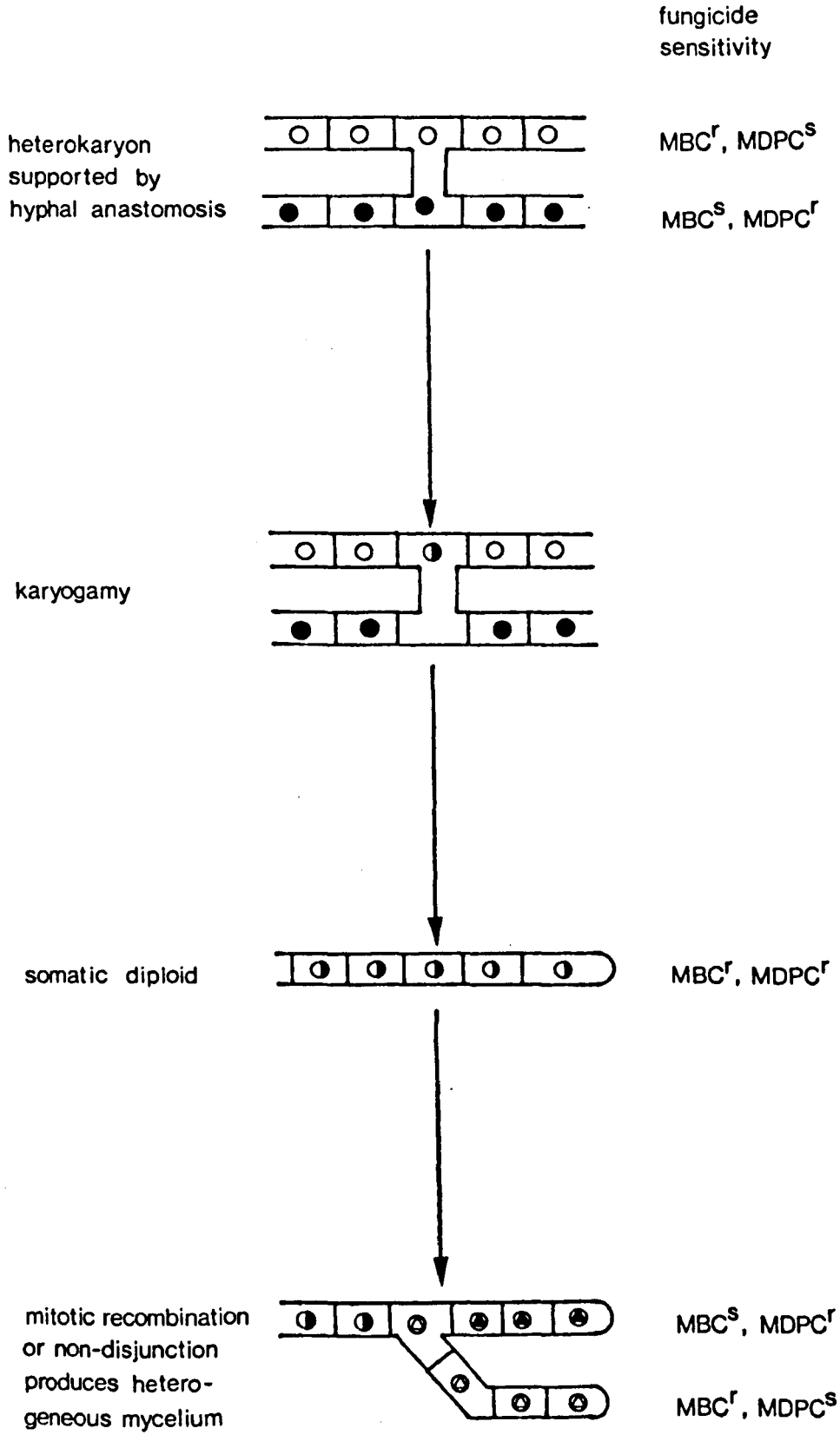
Since the ploidy level of the recombinant auxotrophic progeny obtained in this study has not been determined, and the linkage relationships of the markers used are unknown, it is not possible to state which of these processes are responsible for their production. In several instances, however, colonies already expressing one or more parental markers were found to segregate for the remaining markers in the cross.

Resistance to carbendazim or MDPC appears to behave as a semi-dominant character, selection on a high concentration of either compound favouring the appropriate resistance gene. Re-testing mycelium taken from recombinant auxotrophic colonies, apparently doubly resistant to carbendazim and MDPC, often

yielded colonies sensitive to one or other fungicide. This suggests that such doubly resistant colonies may actually be heterogeneous, containing mycelium with nuclei conferring either parental resistance phenotype, with culture in the presence of one or other compound selecting for the resistant population (fig 6.19).

This theory, however, does not explain the slow, irregular growth of some colonies on fungicide-containing medium, nor the occasional recovery of colonies seemingly sensitive to both inhibitors. Consequently, although reversion studies and much of the genetic evidence supports the idea of a single gene conferring reciprocal resistance and sensitivity to carbendazim and MDPC, further study is required before firm conclusions can be drawn.

Figure 6.19 Diagrammatic representation of parasexual recombination in P. herpotrichoides. Anastomosis bridges support the growth of heterokaryotic mycelium on a selective medium. Random nuclear fusion between dissimilar nuclei produces heterozygous diploid nuclei, which may divied to produce diploid mycelium. Segregation of parental markers occurs as a result of mitotic recombination and/or non-dis-junction in diploid nuclei, and produces "mixed" colonies with nuclear heterogeneity. If the parental strains differed in fungicide respoonse, transfer of heterogeneous mycelium to fungicide-containing medium will select for the resistant nuclear population.


fungicide sensitivity
heterokaryon supported by hyphal anastomosis
MBC^r, MDPC^s
MBC^s, MDPC^r
karyogamy
somatic diploid
MBC^r, MDPC^r
mitotic recombination or non-disjunction produces hetero-geneous mycelium
MBC^s, MDPC^r
MBC^r, MDPC^s

CHAPTER 7

HETEROKARYON INCOMPATABILITY IN *PSEUDOCERCOSPORELLA*

7.1 INTRODUCTION

Gene exchange between different individuals in a population can only occur when the genetic material of those strains is brought together in the same cytoplasm. Hyphal anastomosis is naturally the first step in this process. Any system which prevents hyphal fusions between particular isolates will obviously limit the extent to which natural genetic reassortment can take place. Vegetative or heterokaryon incompatibility has been described in a number of fungi including some important plant pathogens (Caten & Jinks, 1966; Tinline & MacNeill, 1969; Genovesi & MaGill, 1976; Anderson, 1982; Leach & Yoder, 1983; Sidhu, 1983; Hastie & Heale, 1984).

Compatible hyphal fusion events have usually been detected using complementary recessive genetic characters, typically auxotrophic or pigmentation mutants; complemented heterokaryotic growth having a non-parental phenotype (Anagnostakis, 1982; Anagnostakis *et al*, 1986; Puhalla, 1984a, b). However, the introduction of appropriate markers in the large number of isolates necessary to assess the diversity of compatibility relationships is time-consuming. To circumvent this problem positive selection systems for the isolation of suitable auxotrophic mutants have been used in several studies. In these systems spontaneous mutations for resistance to a particular drug (chlorate, selenate, fluoroacetate, *p*-fluorophenylalanine) is

often associated with an auxotrophic requirement (Apirion, 1962, 1965; Arst, 1968; Cove, 1976a, b; Teow & Upshall, 1983). Selection for resistance, which is easily performed, can therefore be used for the rapid isolation of spontaneous, auxotrophic mutants from field isolates.

Complementary auxotrophic strains, obtained by selection for resistance to chlorate, have been used to test for vegetative incompatibility in Fusarium oxysporum (Puhalla 1985; Correll et al, 1986,), Gibberella fugikuroi (Puhalla & Speith, 1985; Sidhu, 1986) and Septoria nodorum (Newton & Caten, 1985). These mutants require a source of reduced nitrogen for normal growth on a defined medium. Three classes of auxotroph are obtained by this method, as a result of mutation in four genes involved in stages of the nitrate reduction pathway (Fig. 7.1). These are nia mutants, deficient in nitrate reductase, cnx mutants deficient for a molybdenum co-factor required by the nitrate reductase enzyme, and nir mutants in which both reductase enzymes are deficient as a result of mutation in a regulatory gene. All three types are mutually complementary (Cove, 1976a, b; Birkett & Rowlands, 1981).

Reduced sulphur-requiring auxotrophs can be produced by selection for resistance to sodium selenate (Arst, 1968). These may result from mutation in one of four loci in A. nidulans, associated with uptake and reduction of sulphate to sulphite (Fig. 7.2).

Both selection systems were used to isolate auxotrophic mutants from a range of Pseudocercosporella isolates. These mutants were then tested for their ability to produce

Figure 7.1 Mutations obtained by selection for resistance to chlorate affecting nitrogen metabolism in Aspergillus nidulans (After Cove 1976a,b).

Enzymes:

1 nitrate reductase

2 nitrite reductase

3 xanthine dehydrogenase

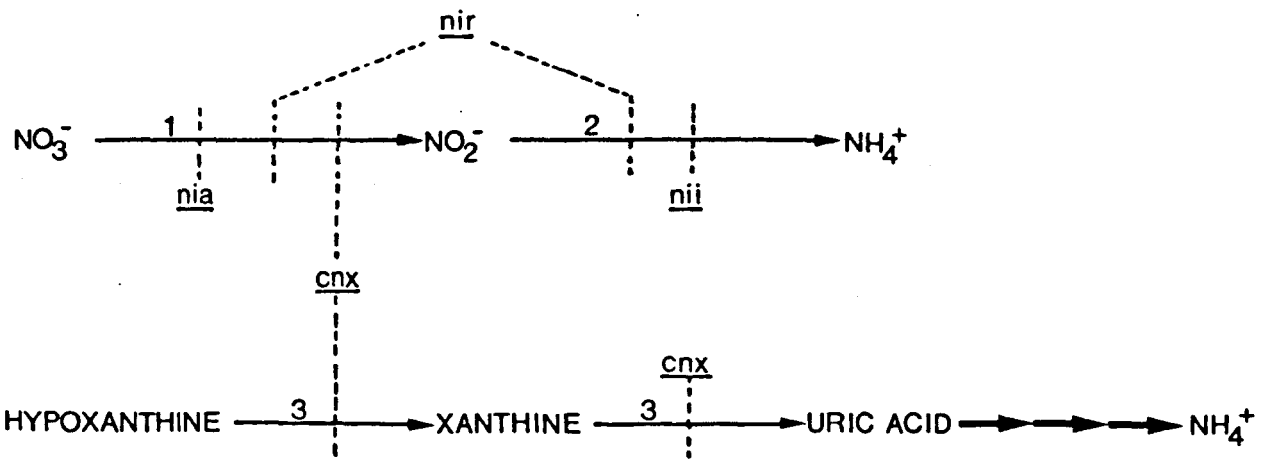

nir
NO_3^-
1
NO_2^-
2
NH_4^+
nia
nii
cnx
cnx
HYPOXANTHINE
3
XANTHINE
3
URIC ACID
NH_4^+

Figure 7.2 Mutations obtained by selection for resistance to selenate affecting sulphur metabolism in Aspergillus nidulans (After Arst, 1968).

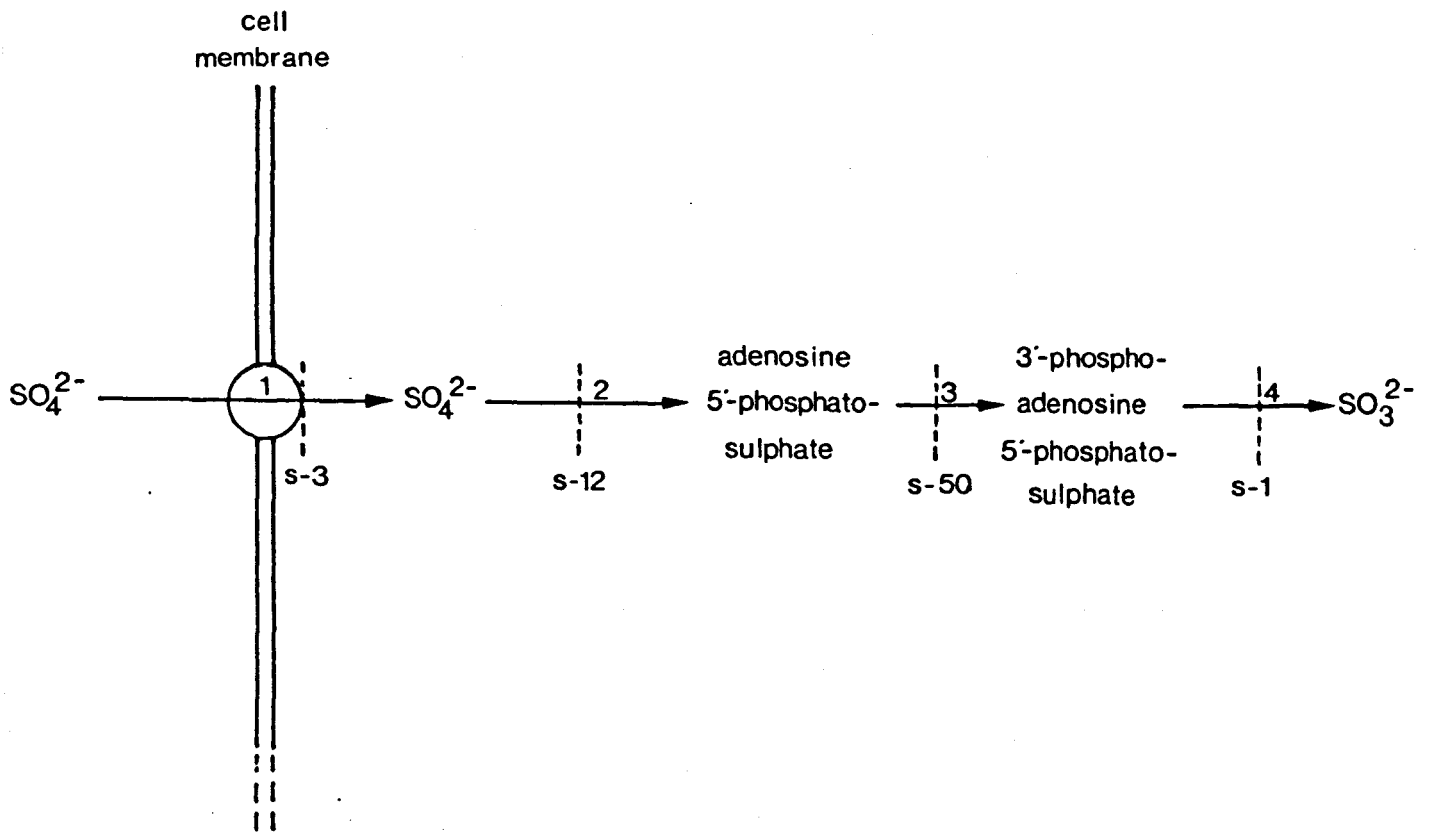
Enzymes:

1 sulphate permease

2 sulphate adenyltransferase

3 adenyl sulphate kinase

4 PAPS reductase


cell
membrane
SO_4^{2-}
1
s-3
SO_4^{2-}
2
s-12
adenosine
5'-phosphato-
sulphate
3
s-50
3'-phospho-
adenosine
5'-phosphato-
sulphate
4
s-1
SO_3^{2-}

heterokaryotic growth on MM. In this way a picture of the vegetative compatibility relationships in this fungus was constructed.

7.2 MATERIALS AND METHODS

7.2.1 Strains

Reduced nitrate- and reduced sulphur-requiring mutants were induced in 3 BW-type isolates (22.20, 22-22, 22-115) and 9 BWR-type isolates (22-6, 22-7, 22-8, 22-9, 22-13, 22-14, 22-15, 22-16, 22-120). Other isolates included 22-8/1, a fast growing strain derived as a morphological variant sector from 22-8, P. h. var acuformis isolate 22-116 and P. aestiva isolate 23-1. Isolates with other auxotrophic or morphological markers were 22-109 (adeA-1) and 22-126 (sB-2), auxotrophic mutants derived from the BW-type isolate 22-20; 22-186 (lys-5) and 22-228 (s-71), auxotrophic mutants from the BWR-type isolate 22-12, and 22-256 (spi-5) a spontaneous, morphological mutant with spiral growth of isolate 22-22. In addition, marked strains were tested in combination with 22-1, a BW-type isolate with a naturally occurring auxotrophic requirement.

7.2.2 Isolation of auxotrophic resistance mutants.

Selenate-resistant mutants were obtained by plating a mixture of spores and hyphal fragments, produced on TWA, on MM containing 25 mM sodium selenate. A proportion of the plates were supplemented with homocysteine thiolactone (0.05 mg ml^{-1}), as an alternative sulphur source. Colonies appeared over a period of 59

days on both series of plates. These were purified in the usual way and single colonies tested for auxotrophy by plating on MM and MM supplemented with cysteine. Auxotrophic mutants were tested for intra-isolate complementation on MM, in order to differentiate different s^- mutations. Those isolated in 22-20 were also tested against the other $\underline{s}^-$ mutants of this isolate (Chapter 4.0).

Reduced nitrogen-requiring mutants were produced by plating on MM containing 245 mM potassium chlorate, with or without arginine 0.05 mg ml^{-1} as an alternative nitrogen source. Chlorate-resistant colonies were more difficult to identify. Addition of arginine resulted in considerable background growth which prevented the recognition of possible resistant forms. On chlorate-containing MM without arginine, resistant mutants formed small dense colonies that differed from the background growth principally in pigmentation, appearing grey rather than reddish-brown. These colonies were purified on MYG agar and individual colonies assessed for growth on MM supplemented with or without arginine. Chlorate-resistant auxotrophs were classified as *nia*, *nir* or *cnx* on the basis of their growth on nitrite, hypoxanthine and ammonium (Table 7.1). Auxotrophic mutants produced by both selection systems were leaky for growth on MM. Pigmentation on MM was generally poorly developed and mycelial growth relatively sparse.

7.2.3 Assessment of compatibility for heterokaryon formation.

Mutants with potentially complementary requirements were assessed for their ability to produce heterokaryotic growth on

Table 7.1 Classification of chlorate-resistant auxotrophic mutants as mutations in nitrate reductase (*nia*), nitrate regulatory (*nir*) or molybdenum co-factor (*cnx*) genes.

	GROWTH ON MM PLUS*:			
GENOTYPE	NO_3^-	NO_2^-	HPX	NO_2^-+NH_4^+
Wild-type	+	+	+	+
nia	-	+	+	+
nir	-	-	+	-
cnx	-	+	-	+

* NO_3^- = sodium nitrate, 0.5 mg ml^{-1}; NO_2^- = sodium nitrite, 0.05mg ml^{-1}; HPX = hypoxanthine, 0.05 mg ml^{-1}; NH_4^+ = ammonium sulphate, 0.005 mg ml^{-1}; - = poor growth; + = normal growth.

MM. Where markers permitted intra- as well as inter-isolate combinations were tested. Auxotrophs of each isolate were point inoculated on MM, 10 mm apart. The plates were incubated at 19° C for up to 46 days, to allow the hyphal fronts of the mutants to meet; heterokaryotic growth was clearly recognisable as zones of darkly pigmented mycelium at junction of complementary colonies. Non-complementary combinations did not produce these zones, suggesting that there was no significant cross-feeding between incompatible isolates.

7.2.4 Chemicals

Potassium chlorate and sodium selenate were purchased from BDH. Both were prepared as stock solutions in water, sterilized by filtration through cellulose nitrate membranes (Pore size = 0.02 μm), and added to the molten agar medium, after autoclaving.

7.3 RESULTS

7.3.1 Mutant Isolation and Characterisation

Selenate-resistant, reduced sulphur-requiring auxotrophic mutants were obtained from 14 isolates and chlorate-resistant auxotrophs from 4 isolates, including 22-8/1 (Table 7.2). Within each isolate none of the selenate selected mutants obtained were complementary, suggesting that they all belonged to

Table 7.2 Auxotrophic mutants obtained by selenate and chlorate selection of wild-type isolates.

ISOLATE TYPE	NUMBER	Number of auxotrophs selected on: SELENATE	CHLORATE		
		s⁻	*nia*⁻	*nir*⁻	*cnx*⁻
BWR	22-6	8	-*	-	-
	22-7	1	-	-	-
	22-8	6	8	0	2
	22-9	3	-	-	-
	22-13	1	-	-	-
	22-14	-	3	0	1
	22-15	1	-	-	-
	22-16	8	-	-	-
	22-120	-	1	1	0
BW	22-20	12	-	-	-
	22-22	1	-	-	-
	22-115	1	-	-	-
BWR variant	22-8/1	1	4	0	1
P.h. var *acuformis*	22-116	7	-	-	-
P.aestiva	23-1	14	-	-	-

* Not selected on this compound

the same mutant class. The chlorate selected auxotrophs were readily classified as *nia*, *nir* or *cnx*; most were of the first type. Since no complementation tests between mutants of similar

phenotype were conducted the number of loci involved is not known.

7.3.2 Inter-Strain Compatability

Most combinations of isolates failed to produce complemented growth on MM (Table 7.3). In some cases there was no contact between the hyphal fronts of the paired colonies, presumably due either to competition for nutrients or antagonistic interactions. For the majority of combinations

Table 7.3 Heterokaryon compatibility in isolates of Pseudocercosporella. Pairs of strains with auxotrophic markers were tested for complementation on MM after 59 days at 19°C.

	ISOLATE NUMBER							
ISOLATE NUMBER	22-20	22-12	22-8	22-8/1	22-14	22-120	22-1	22-22
22-20	+	-	-	-	-	-	-	-
22-12	-	+	+	+	-	-	-	-
22-8	-	+	+	+	-	-	-	-
22-8/1	-	+	+	+	-	-	-	-
22-14	-	-	-	-	?	-	-	-
22-120	-	-	-	-	-	?	-	-
22-1	-	-	-	-	-	-	NA	-
22-22	-	-	-	-	-	-	-	+
22-6	-	-	-	-	-	-	-	-
22-7	-	-	-	-	-	-	-	-
22-9	-	-	-	-	-	-	-	-
22-13	-	?	-	-	-	?	-	-
22-15	?	-	-	-	-	?	-	-
22-16	?	?	-	-	-	-	-	-
22-115	-	-	-	-	-	-	-	+
22-116	-	-	-	-	-	-	-	-
23-1	-	-	-	-	-	?	-	-

- = no complementation; + = complementation for growth; NA = not tested; ? = no contact between hyphae of different isolates.

hyphal contact was observed, and failure to produce heterokaryotic growth was taken to indicate vegetative incompatibility between the two isolates.

Compatible interactions were seen between mutants derived from the same isolate and also between certain unrelated isolates (Fig. 7.3). Compatible inter-strain interactions occurred between the BW-types 22-22 and 22-115, and between the BWR-types 22-12 and 22-8. Heterokaryon compatibility was also seen between 22-8 and the morphological variant of this isolate 22-8/1, and between 22-8/1 and isolate 22-12.

7.3 DISCUSSION

The large number of non-complementary interactions suggests that heterokaryon incompatibility is widespread in P. herpotrichoides. If this phenomenon also occurs under natural conditions these findings have obvious implications for potential genetic exchange in the field.

Vegetative incompatibility is common in Septoria nodorum, most pairs of isolates tested were unable to form heterokaryons in culture (Newton & Caten, 1985). Compatible reactions were only found amongst strains originating from the same fields, and therefore possibly clones. Two pieces of evidence suggest that the compatible interactions observed in P. herpotrichoides in this study were between unrelated field isolates. Firstly they originated from different cereal hosts and isolates 22-22 and 22-115 were obtained at different times. Secondly the benzimidazole sensitivities of 22-12 and 22-8 were different, 22-12 being

carbendazim-sensitive and 22-8 highly carbendazim-resistant. In addition, when cultured on MYG agar all four strains were morphologically dissimilar.

The positive reaction of isolate 22-12 with 22-8/1 confirms that this variant was derived from isolate 22-8. Unfortunately because of the similarity of markers carried by many of the strains a full comparison of compatibility between all isolates was not possible. However, none of the other BWR-types was able to complement either 22-12, 22-8 or 22-8/1. Whether these isolates form a single mutually compatible group awaits the isolation and testing of further mutants.

No compatible reactions were observed between any of the P. herpotrichoides strains and 23-1, the P. aestiva isolate. These results confirm the findings of Nirenberg (1984), who was unable to detect hyphal anastomosis between P. herpotrichoides strains and the other Pseudocercosporella species, or between the two taxonomic varieties of P. herpotrichoides.

CHAPTER 8

PATHOGENICITY STUDIES

8.1 INTRODUCTION

Numerous methods have been devised to measure pathogenicity of fungal pathogens. These vary depending on the nature of the disease and the symptoms that are produced, and may be direct (eg. leaf area infected, lesion size, number of infections) or indirect (eg. degree of stunting, yield reduction) assessments. Where quantitative assessments are difficult to make, as for example with wilting or epinasty, severity of symptom development may be estimated.

Eyespot disease, caused by P. herpotrichoides, has been assessed in several ways. In seedling tests mean penetration scores, calculated from the number of leaf sheaths infected by the fungus, have been widely used (Scott, 1971; Law et al, 1976; Scott et al, 1976; Bateman et al; 1985). Assessment of lesion development is generally preformed 4 to 12 weeks after inoculation. In mature plants simpler scoring systems, based on the degree of stem girdling, have been devised (Lange-de la Camp, 1966b; Bruehl & Cunfer, 1972; Scott & Hollins, 1974; Bruehl et al, 1982; Murray & Bruehl, 1983). In which case disease assessments are made at the end of the growing season, normally 6 to 7 months for winter cereal cultivars. These scores are usually converted into a disease index in which more severe infections are given greater weighting. Higgins and Fitt (1984, 1985) attempted to combine the two approaches by including both penetration and girdling scores in a single disease index, while

the proportion of tillers affected in each plant was reflected in the penetration and severity indicies of Higgins et al (1986). Yield components, including total harvestable yield, yield per head and 1000 grain weight, have been measured to determine the economic impact of infection (Davies & Jones, 1970). In addition, the disease incidence (ie. the percentage of plants and/or tillers infected) has been used to estimate the amount rather than the severity of disease present (Davies & Jones, 1970; Hollins & Scott, 1980).

All these systems are time consuming and laborious. More rapid tests involving microscopic examination of in vivo mycelial growth, appressoria formation, the number of penetration sites and papillae formation by the host have been used to investigate host resistance to infection (Guillot-Salomon et al, 1981; Kahn & Bouriquet, 1984; Kahn et al, 1986; Murray & Ye, 1986). However, these methods do not appear to have been applied to the compar-ison of different fungal isolates. De Virville et al (1982) described a technique for the rapid determination of pathogenicity and host resistance in which the level of respiration of host tissue was measured. Clear differences in response were observed between an eyespot-suseptible and an eyespot-resistant wheat cultivar inoculated with pathogenic or non-pathogenic isolates of the fungus. The ability of this test to disciminate between isolates differing only slightly in pathogenicity requires further study.

Inoculation procéedures have been equally diverse. Spores are the usual source of infection in the field, produced on debris from previous crops. Several methods have been used which

attempt to mimic these events. Straws or oat kernals, infested with the fungus and placed on the soil surface where they generate conidia during cool, wet weather, are the usual source of inoculum in long term pathogenicity tests (Bruehl & Cunfer, 1972; Scott & Hollins, 1974; Guillot-Salomon et al, 1981; Bruehl et al, 1982; Murray & Bruehl, 1983, 1986; Higgins & Fitt, 1984; Higgins et al, 1986). Alternatively, suspensions of spores or homogenised mycelium can be sprayed onto the plants, although inoculum applied in this way is more susceptible to dessication (Lange-de la Camp, 1966b; Davis & Jones, 1970; Murray & Bruehl, 1983; Hoare ,1986).

A different approach, which has often been used to inoculate plants in seedling tests, was described by Macer (1966). Straw segments infested with the fungus are placed over the emergent coleoptile, and covered with either sand or soil to maintain humidity, mycelium from the straw is then able to infect the plant tissues directly. Modifications of this technique include the use of agar collars in which mycelium has been embedded, in place of straw, thereby reducing the time required for production of the inoculum (Bateman et al, 1985). Filter paper disks containing either mycelium or conidia have also been successfully substituted for straw collars (Evans & Rawlinson, 1975; Kahn & Bouriquet, 1984; Higgins & Fitt, 1984, 1985).

Comparisons of the pathogenicities of various isolates of P. herpotrichoides to different cereal hosts identified two main pathogenicity types of the fungus (Lange-de la Camp, 1966b; Scott et al, 1975). These pathotypes (W or BW and R or BWR) were

equally pathogenic to wheat plants but differed in their pathogenicity to rye, BWR-types being more pathogenic to this host. The association of pathotype with cultural morphology is used generally as a character to differentiate BW- and BWR-type isolates in field surveys of the fungus. Such studies have revealed that changes in the relative proportions of the two types occur in field populations of the pathogen, both during the season and with different crop species (Griffin, 1985; Bateman et al, 1985). However, many isolates are difficult to classify as belonging to either morphological type. This together with the instability of the BWR growth form in culture makes precise monitoring of these changes difficult.

Readily recognisable characters, which do not seriously impair pathogenicity, are required. Most auxotrophic mutations may be expected to reduce pathogenicity. Pigmentation and resistance mutations, however, may be less deleterious and so suitable for use as markers in pathogenicity studies. The pathogenicity of isolates with differing cultural morphology to a range of cereal hosts was determined. In addition, the effect on pathogenicity of various mutations was assessed.

8.2 MATERIALS AND METHODS

8.2.1 Strains

The origin and details of the field isolates used in pathogenicity tests are given in Appendix 1. The BW-type isolates assessed were 22-2 and 22-20, BWR-type isolates were 22-8, 22-10, 22-12 and 22-117. In addition the pathogenicities of the P. h.

var. acuformis isolate, 22-116, and the P. aestiva isolate were determined. Lysine-requiring auxotrophic mutants derived from isolates 22-20 (22-127; lysB-4, ben-17) and 22-12 (22-186; lys-5), and a sporulation mutant (22-184; con-2) derived from 22-12 were also screened.

8.2.2 Host Plants

Five cereal cultivars were used to screen pathogenicity of P. herpotrichoides isolates: two cultivars of winter wheat, 'Armada', an eyespot-susceptible cultivar (NIAB resistance rating 4), and 'Rendezvous', a relatively resistant cultivar containing eyespot resistance derived from Aegilops ventricosa (T.W. Hollins pers. comm.), one winter barley cultivar, 'Pirate'; a winter rye cultivar, 'Animo' and 'Newton, a cultivar of triticale. All cereals were obtained from the National Institute for Agricultural Botany (NIAB), Cambridge except 'Rendezvous' which was provided by Dr. T.W. Hollins of the Plant Breeding Institute, Cambridge.

8.2.3 Inoculation Protocol

The inoculation procedure was similar to that described by Macer (1966). Fungal strains were grown on 2 to 3 cm internodal pieces of wheat straw, in Woods flasks, for approximately 4 weeks at 19°C. Straw cultures were kept damp by the periodic addition of 5 ml liquid MM, supplemented, in the case of auxotrophic mutants, with 0.4 mg ml^{-1} yeast extract.

Cereal grains were presoaked for 2 h and germinated on damp blotting paper in a moist chamber for 3 to 7 days. Germinated seeds were transferred to 9 cm pots containing peat-

based compost, sterilised by autoclaving at 121°C for 30 min. Pots were filled to within 2 cm of the top. Straw collars, infested with the fungus, 1 cm long, were placed over the emergent coleoptiles, and the inoculated pots filled with sterile sand. Four seedlings were placed in each pot, and four pots were inoculated with each isolate. Control pots of each cereal were left uninoculated.

Pots containing seedlings were randomly arranged and maintained in a cool glasshouse for 15 weeks (Experiment 1 - 10th January to 29th April, 1986; Experiment 2 - 27th January to 13th May, 1986). Compost was kept damp by regular watering, and the plants fed twice with Hoaglands nutrient solution (Hoagland, 1944). Disease symptoms were assessed according to the system of Scott (1971) as follows:

0 = no infection

1 = coleoptile infected

2 = coleoptile penetrated

3 = first leaf sheath infected

4 = first leaf sheath penetrated

5 = second leaf sheath infected

6 = second leaf sheath penetrated

etc.

Mean penetration figures were calculated as the average of the disease scores for each host-isolate combination.

8.3 RESULTS

Two separate experiments to compare pathogenicity of isolates were performed. In the first, four strains of the fungus, two BW-types and two BWR-types, were screened against the five cereal hosts. Nine isolates, including three mutant strains, were tested in the second experiment. Disease assessments for both experiments were conducted 15 weeks after inoculation. This extended period, compared with the duration of most seedling tests, was necessary because of the unusually prolonged cold weather during February 1986.

Assessment of the extent of infection by the fungus of the wheat cultivar Rendezvous was precluded by extensive browning of the base of the stem, unrelated to the presence of the eyespot fungus. This dicolouration was exhibited by the majority of the seedlings of this cultivar, including most of the uninoculated control plants (Fig 8.1). As a result, the mean disease scores obtained were similar for the inoculated and the control plants. Consequently the results for 'Rendezvous' were ommitted from the analysis of the data from the other cereal hosts. Attempts to reisolate the eyespot pathogen from the inoculated 'Rendezvous' plants were unsuccessful. A fast-growing, unidentified fungus was recovered from most surface sterilised, leaf sheath segments, though whether the presence of this fungus was the cause of the discolouration of the stem bases is not known.

8.3.1 Field Isolates

Significant differences in pathogenicity were found between isolates in both experiments (Fig. 8.4). In addition host cultivars were infected to differing extents and statistical analysis revealed a significant degree of interaction between isolates and hosts (Tables 8.1, 8.2).

Table 8.1 Analysis of variance of pathogenicity results from experiment 1. Four isolates, 22-2, 22-10, 22-12 and 22-20 were screened for differences in pathogenicity against 'Armada' wheat, 'Pirate' barley, 'Animo' rye and 'Newton' Triticale.

SOURCE	DF	SS	MS	F	
Isolates	3	441.359	147.120	29.73	P = 0.001
Cultivars	3	2191.591	730.530	147.61	P = 0.001
Interaction	9	158.340	17.593	3.55	P = 0.001
Residual	223	1103.654	4.949		
Totals	238	4373.155			

On the winter wheat cultivar Armada only small differences in pathogenicity were observed between most isolates. Isolate 22-117, morphologically a BWR-type, was significantly less pathogenic (P = 0.001) than all other field strains tested in experiment 2, this difference was also apparent on the other host species. 'Animo', the rye cultivar, was relatively resistant to infection by the fungus, consequently differentiation of BW- and BWR-type isolates on the basis of pathogenicity to rye was not reliable. However, in both experiments, the highest penetration

Figure 8.1 Wheat 'Rendezvous' plants showing stem browning not associated with Pseudocercosporella infection. Left to right: uninoculated control, inoculated with 22-8 (BWR), inoculated with 22-20 (BW).

Figure 8.2 Barley 'Pirate' plants showing differential severity of infection. Left to right: uninoculated control, 22-2 (BW), 22-8 (BWR), 22-20 (BW), 22-116 (P.h. var. acuformis).

Figure 8.3 Wheat 'Armada' plants inoculated with different Pseudocercosporella isolates. Left to right: uninoculated control, 22-2 (BW), 22-8 (BWR), 22-20 (BW), 22-116 (BWR), 22-184 (con-2), 23-1 (P. aestiva).

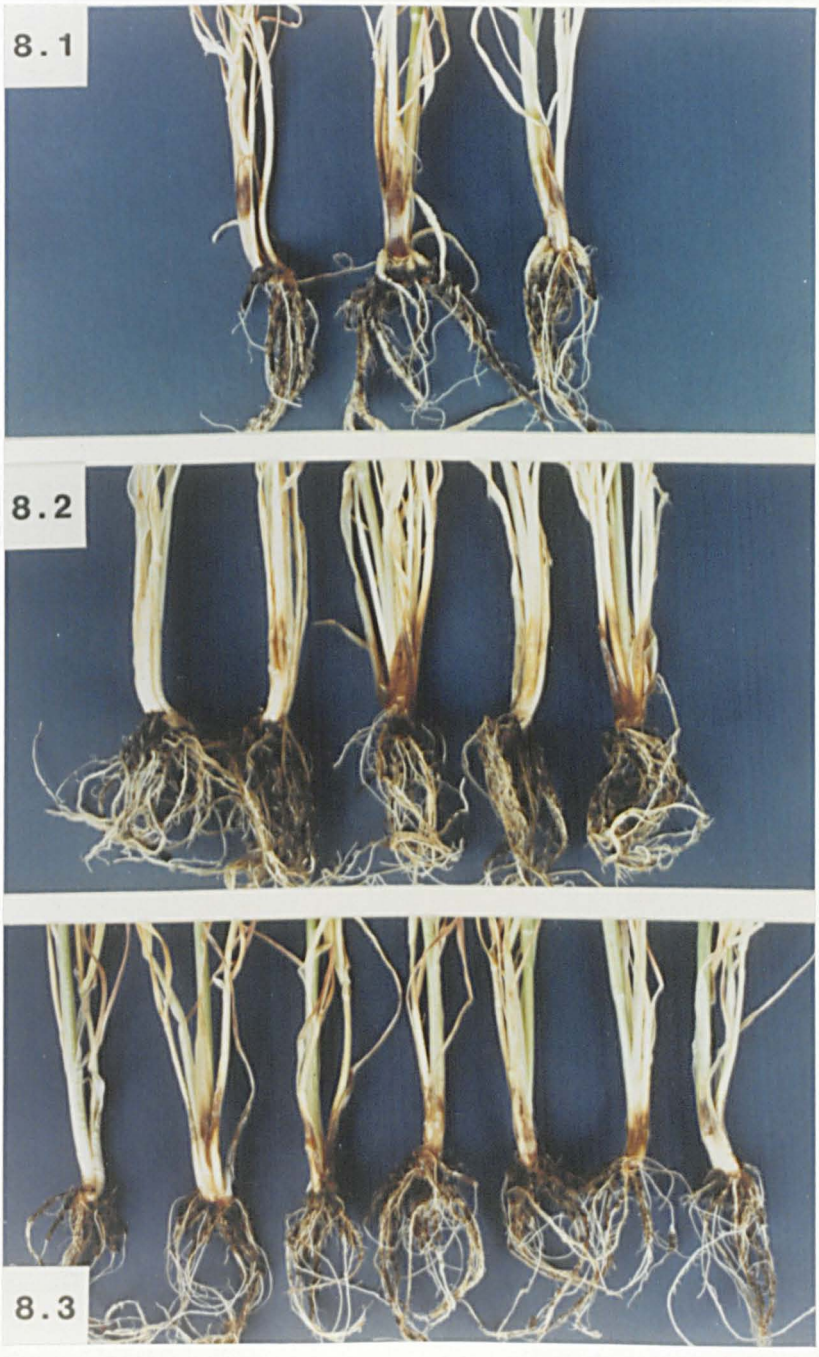
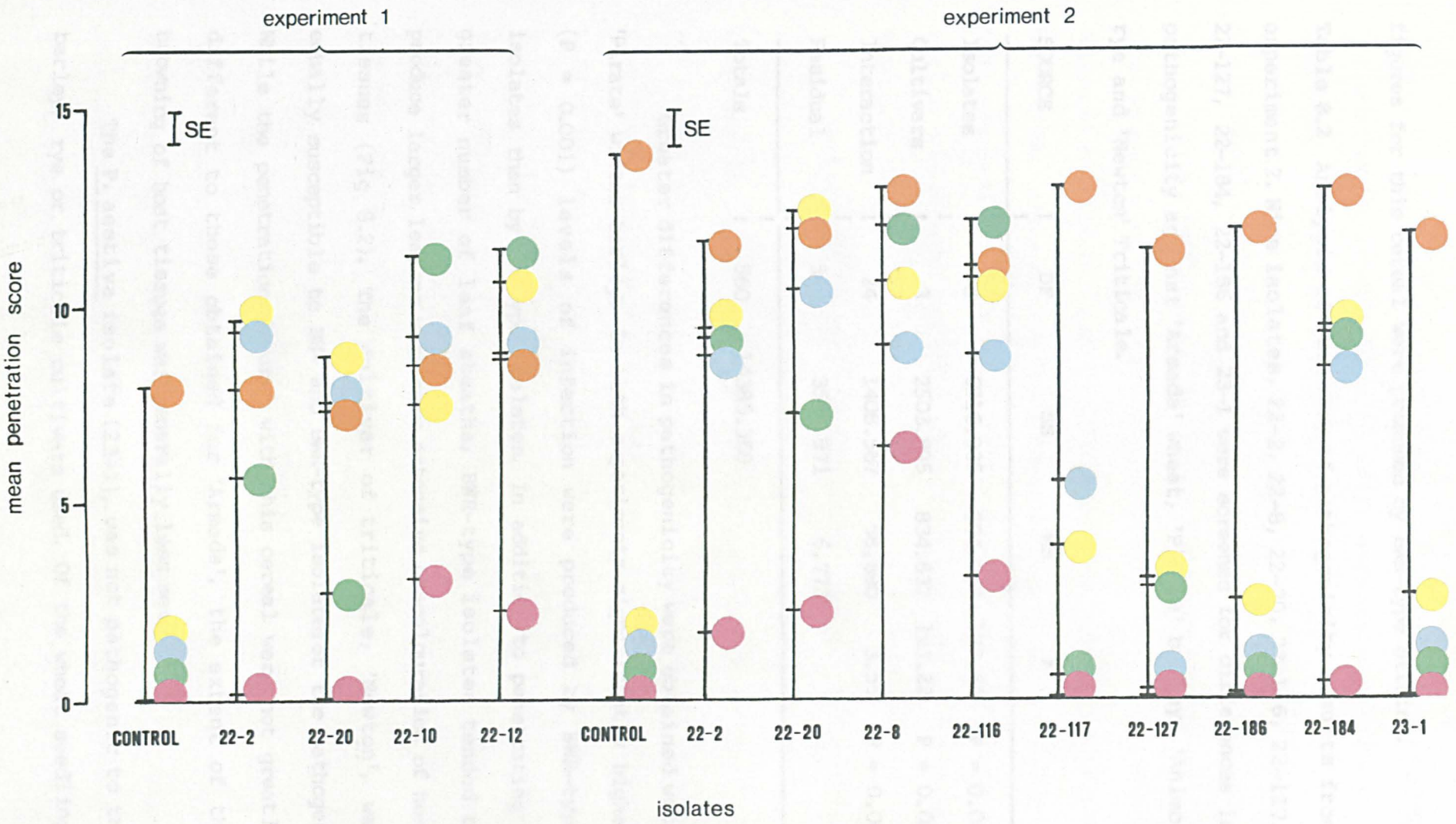

8.1
8.2
8.3

Figure 8.4 Mean penetration scores for isolates on a range of cereal hosts. Plants assessed 15 weeks after inoculation with straw infested with the fungus. Standard error for differences between two values calculated for each experiment, omitting data for wheat cultivar Rendezvous. = 'Armada' wheat; = 'Rendezvous' wheat; = 'Pirate' barley; = 'Animo' rye; = 'Newton' triticale.


experiment 1
experiment 2
SE
SE
15
10
5
0
mean penetration score
CONTROL
22-2
22-20
22-10
22-12
CONTROL
22-2
22-20
22-8
22-116
22-117
22-127
22-186
22-184
23-1
isolates

figures for this cereal were produced by BWR-type strains.

Table 8.2 Analysis of variance of pathogenicity results from experiment 2. Nine isolates, 22-2, 22-8, 22-20, 22-116, 22-117, 22-127, 22-184, 22-186 and 23-1 were screened for differences in pathogenicity against 'Armada' wheat, 'Pirate' barley, 'Animo' rye and 'Newton' Triticale.

SOURCE	DF	SS	MS	F	
Isolates	8	6916.935	864.617	127.65	P = 0.001
Cultivars	3	2503.895	834.632	123.22	P = 0.001
Interaction	24	1408.567	58.690	3.55	P = 0.001
Residual	525	3555.971	6.773		
Totals	560	14385.368			

Greater differences in pathogenicity were obtained with 'Pirate' winter barley. In both experiments significantly higher (P = 0.001) levels of infection were produced by BWR-type isolates than by BW-type isolates. In addition to penetrating a greater number of leaf sheaths, BWR-type isolates tended to produce larger lesions with more extensive discolouration of host tissues (Fig 8.2). The cultivar of triticale, 'Newton', was equally susceptible to BW- and BWR-type isolates of the pathogen. While the penetration figures with this cereal were not greatly different to those obtained for 'Armada', the extent of the browning of host tissues was generally less severe.

The P. aestiva isolate (23-1), was not pathogenic to the barley, rye or triticale cultivars used. Of the wheat seedlings

inoculated with this isolate few had visible signs of infection, characteristic eyespot lesions were, however, produced on a proportion of the plants (Fig. 8.3).

8.3.2 Mutant strains

The pathogenicity of the lysine auxotrophs, 22-127 and 22-186, was much reduced. Slight infection by both strains was observed on 'Armada', and 22-127 caused some disease on 'Pirate' barley. The levels of infection, however, were significantly less than the isolates from which they were derived (fig. 8.4). No lesion development was observed with these strains on either Triticale or rye.

In contrast 22-184, the sporulation mutant derived from isolate 22-12, retained the ability to cause typical disease symptoms. On 'Armada' the penetration score for this mutant was not significantly different ($P = 0.05$) to those obtained with the four field isolates tested, while the level of infection of barley was equivalent to that produced by isolate 22-2. Pathogenicity to triticale was also equal to that of the wild type isolates. This mutant was non-pathogenic on the rye cultivar, 'Animo'. The pathogenicity of isolate 22-12 on rye, however, was only slight.

8.4 DISCUSSION

The use of straw segments infested with the fungus to inoculate cereal seedlings successfully produced lesions characteristic of eyespot disease. Scott (1971) calculated that to reduce the experimental error inherent in seedling tests of

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8.4 DISCUSSION

The use of straw segments infested with the fungus to inoculate cereal seedlings successfully produced lesions characteristic of eyespot disease. Scott (1971) calculated that to reduce the experimental error inherent in seedling tests of

this type to a level which allowed accurate detection of differences in host response, at least fifty-two plants would be required for each isolate-host combination. This degree of replication was not possible in the experiments conducted in the present study, due to a lack of sufficient glasshouse space. Nevertheless, clear differences in pathogenicity between isolates were detected.

The rye cultivar, 'Animo', was not a suitable host for the differentiation of the two pathotypes. Although the BWR-type isolates produced higher disease scores than BW-types, the levels of infection were generally very low. The two types could, however, be ditinguished by their differential pathogenicity to 'Pirate' barley. BWR-type isolates being substantially more pathogenic on this host than BW-types. These results are similar to the findings of King & Griffin (1985), and may explain the increasing preponderance of BWR-type isolates in areas where barley is grown as an alternative to wheat.

Isolate 22-116, the *P. h.* var. *acuformis* isolate, was similar in pathogenicity response to the BWR-type isolates tested. While caution is obviously necessary when drawing conclusions based on so few isolates, this finding supports the identification of this taxonomic group with the BWR pathotype (King & Griffin, 1985). *P. aestiva* (23-1) was relatively non-pathogenic to the cereal cultivars tested. Nirenberg (1985) reported that this was the least frequently isolated species of *Pseudocercosporella* in Germany. While the low incidence of *P. aestiva* in the field may be due to its relatively low

pathogenicity, a larger number of isolates need to be tested before definite conclusions can be drawn.

The use of genetically marked strains to monitor population changes in the field necessarily requires markers that do not reduce pathogenicity. Neither of the two auxotrophically marked mutants tested in experiment 2 retained significant levels of pathogenicity compared with the isolates from which they were obtained. While many auxotrophic mutations may have a deleterious effect on pathogenicity, the association is not absolute. Some auxotrophic mutants of Verticillium albo-atrum were shown to be equally as pathogenic as wild-type strains (Clarkson & Heale, 1985b), while pathogenicity of auxotrophic mutants of Venturia inaequalis was restored by the addition of the appropriate exogenous amino acids or vitamins (Wood, 1967). Furthermore the identification of an auxotrophic requirement in one of the P. herpotrichoides field isolates (22-1), strongly suggests that nutritional requirements which do not affect pathogenicity in this fungus can be produced.

Morphological mutations such as con-2, which did not greatly reduce pathogenicity in this study, may also prove useful in studies where identification of particular isolates is needed. This mutation has the added advantage of allowing large quantities of spore inoculum to be prepared with relative ease. In addition, such strains may be used to demonstrate parasexual genetic exchange between different strains in vivo.

CHAPTER 9

GENERAL DISCUSSION

Several aspects of the biology of the eyespot pathogen, P. herpotrichoides are particularly interesting and merit further investigation. Pathogenicity and host specificity differences between isolates of the fungus have been correlated with differences in in cultural morphology (Lange-de la Camp, 1966; Scott et al 1975). Instability of the morphological character, particularly noticeable in BWR-type strains, raises important questions about the status of the two major pathogenic forms and their inter-relationship. Since much emphasis has been placed on this morphological distinction between the two pathotypes, in field surveys of both fungicide resistance and seasonal changes in the population structure (Hollins et al, 1985; King & Griffin, 1985), it is vital that the significance and extent of the cultural variation in the pathogen be clarified. In this task additional characters including biochemical markers (total protein profifes, isozyme differences, restriction fragment length polymorphisms) may be particularly useful.

The genetic basis of the instability is as yet unclear. However, the use of heterokaryon tests and manipulation of the parasexual cycle should enable the possible involvement of cytoplasmic and nuclear factors to be investigated. In addition it is important that the faster growing variants obtained from BWR-type isolates are screened for changes in pathogenicity against a range of cereal hosts.

Resistance to the benzimidazole fungicides has now become widespread in the eyespot pathogen population. The range

of resistance phenotypes obtainable in the laboratory, has not been observed in resistant forms from the field. This is possibly a consequence of the selection methods used to isolate resistant mutants, but may also be the result of the way field surveys of resistant strains are conducted. The ease with which mutants resistant to both carbendazim and MDPC can be produced in the laboratory, from benzimidazole-sensitive or resistant strains, suggests that the use of phenylcarbamate fungicides, specifically to reduce the frequency of benzimidazole resistant forms in the field, is unlikely to be successful, at least in P. herpotrichoides. A better understanding of the target site of the fungicide and the mechanism of resistance in this fungus, may yet lead to the development of compounds capable of inhibiting carbendazim-resistant strains without the associated risk of dual resistance.

Resistance to the benzimidazole group of fungicides in several species of fungi is known to be altered β-tubulin, the main target site of these compounds. The basis of resistance in P. herpotrichoides is not known but is assumed also to be the result of changes in the tubulin protein structure. This assumption requires confirmation by screening resistance mutants for electrophoretically abnormal tubulin proteins. In this respect, the resistance mutants generated in the course of this study, with the wide range of phenotypes described, will prove particularly useful.

Altered membrane permeability has been shown to be the basis of benzimidazole resistance in some species (Tripathi &

Schlosser, 1982; Welker & Williams, 1983). It is possible that mutants with different levels of resistance identified in the present work represent different resistance mechanisms; the high- and intermediate-level resistance mutants for example, having altered tubulin protein while the low-level resistance mutants result from reduced uptake of the fungicides. It should be possible to differentiate these two classes on the basis of cross-resistance to unrelated inhibitors, as has been shown for Dictyostelium discoideum (Welker & Williams, 1983). Alternatively, culture of the resistance mutants in the presence of compounds known to affect membrane permeability, such as nystatin or the triazole compound miconazole, should eliminate benzimadazole resistance due to decreased uptake while not affecting tubulin-based resistance.

Genetic analysis in P. herpotrichoides is now possible by manipulation of the parasexual cycle, demonstated in this work. The time required for the production of diploids and the recovery of recombinant progeny can be considerably shortened from that described in Chapter 6.0. Heterokaryon formation between compatible strains can be acheived by co-inoculation of the parental strains directly onto the selective medium, either as a mixed conidial suspension or blocks of agar placed in contact on the agar surface. Induced segregation using a haploidizing agent such as p-fluorophenylalanine, rather than fluorouracil, on a medium favourable for spore production, would ensure the rapid recovery of haploid progeny from heterozygous diploid strains , which can then be tested for reassortment of markers in the usual way.

This system of genetic analysis will allow the genetic basis of fungicide resistance to be elucidated. Crosses between strains with different resistance phenotypes will enable the number of genes involoved to be determined and interactions between the phenotypes to be investigated. A fuller examination of suppressor and modifier mutations may prove particularly valuable. Genetic investigation of mutations obtained by selection for revertants in negatively cross-resistant strains should allow additional loci affecting resistance expression to be identified.

The construction of a multiply marked "master strain" would greatly facilitate the mapping of resistance loci. Such a strain could readily be produced by selection for spontaneous resistance to a variety of inhibitors in a strain already carrying auxotrophic markers; thereby avoiding the repeated use of mutagenic agents which can induce major structural chromosome alterations.

Vegetative incompatibility between unrelated strains appears to be common in P. herpotrichoides. Few isolates producing heterokaryotic growth even under selective conditions. Further work, screening a larger number of isolates, is required to establish the extent of this incompatibility and to determine if compatible reactions are resticted to within each pathotype. Protoplast fusion may provide a way by which viable diploids can be produced between vegetatively incompatible isolates. If this is possible, genetic studies of differences in pathogenicity and their association with morphological variation and differential

EBI sensitivity, will greatly assist in the overall characterisation of the organism.

This study of resistance expression and variation in P. herpotrichoides has inevitably raised many more questions than it has answered. The parasexual system described in this work and the collection of mutant strains constructed, however, provide a strong base from which future work can continue.

Appendix I Origin and characteristics of *Pseudocercosporella* field and type isolates

BDUN NUMBER	OTHER NUMBERS	ORIGIN	TYPE	CARBENDAZIM SENSITIVITY
22-1	S1(MD 9)	ADAS (Harpenden)	BW*	S**
22-2	S2(PBI 265)	"	BW	R
22-3	S3(170')	"	BWR	S
22-4	S4(MD 7)	"	BWR	R
22-5		Wheat 'Rapier' (0)***	BW	S
22-6		wheat'Rapier' (0)	BWR	R
22-7		wheat'Rapier' (2)	BWR	R
22-8		wheat'Avalon' (1)	BWR	R
22-9		wheat'Avalon' (2)	BWR	R
22-10		barley'Fenella' (0)	BWR	R
22-11		wheat'Rapier' (0)	BWR	R
22-12		wheat'Rapier' (0)	BWR	S
22-13		wheat'Rapier' (2)	BWR	R
22-14		wheat'Rapier' (1)	BWR	R
22-15		wheat'Rapier' (1)	BWR	R
22-16		wheat'Rapier' (1)	BWR	R
22-17		wheat'Rapier' (1)	BWR	R
22-18	B71/2	Rothamsted Exptl. Stn. (wheat)	BW	S

Appendix I continued

BDUN NUMBER	OTHER NUMBERS	ORIGIN	TYPE	CARBENDAZIM SENSITIVITY
22-19	C71/3	Rothwell Plant Breeders Lincs. (wheat)	BW	S
22-20	C71/8	NIAB, Cockle Park, (wheat)	BW	S
22-21	C71/67	PBI, Cambridge (oats)	BW	S
22-22	C71/73	PBI, Cambridge (rye)	BW	S
22-115	15-4	Rothamsted Exptl. Stn.	BW	S****
22-117	BK 45	Rothamsted Exptl. Stn.	BWR	S
22-118	PBI 265	ADAS (Bristol)	BW	R
22-119	PCB85/ 382/2	ADAS (Bristol)]	BW	R
22-120	PBI 275	ADAS (Bristol)	BWR	S
22-121	170(1)	ADAS (Bristol)	BWR	S
22-182		wheat	BWR	R
22-183		wheat	BWR	R
22-116		Manchester University	*P. h.* var. *acuformis*	S
23-1	64002	Manchester University	*P. aestiva*	S
24-1		FBC Ltd. Chesterford Park	*P. anguoides*	S

Appendix I continued

Isolates 22-19 to 22-22 and infected wheat and barley straw from which isolates 22-5 to 22-17 were isolated were provided by Dr. W. Carlisle, Trent Polytechnic.

* BW Fast-growing, even-edged colony type isolates, pathogenic on barley and wheat.
BWR Slow-growing, irregularly-edged colony type isolates, pathogenic on barley, wheat and rye.

** S Isolates sensitive to carbendazim
R Isolates resistant to carbendazim

*** Previous fungicide history of field:
(0) No previous use of benzimidazole fungicides
(1) Field sprayed only once with benzimidazole fungicides
(2) Field sprayed two or more times with benzimidazole fungicides

**** Spontaneous MDPC-insensitive revertant selected from a carbendazim-resistant field isolate

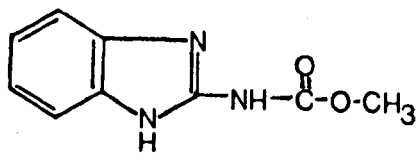
Appendix II Chemical group, structural formulae and µM : µg ml^{-1} concentration conversion tables for fungicides used in this work

Microtubule Inhibitors

$\mu M : \mu g\ ml^{-1}$

Benzimidazoles and Thiophanates

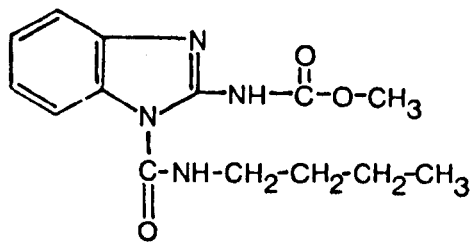
carbendazim mol wt 191.2



10.0 : 1.91

52.3 : 10.0

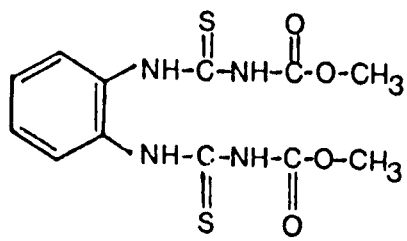
benomyl mol wt 290.3



10.0 : 2.90

34.4 : 10.0

thiophanate-methyl mol wt 342.4

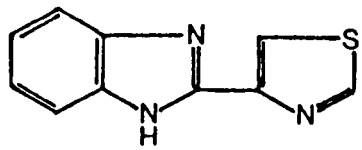


10.0 : 3.42

29.2 : 10.0

μM : $\mu g\ ml^{-1}$

thiabendazole mol wt 201.25

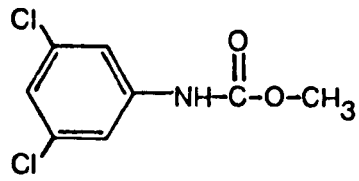


10.0 : 2.01

49.7 : 10.0

N-phenylcarbamates

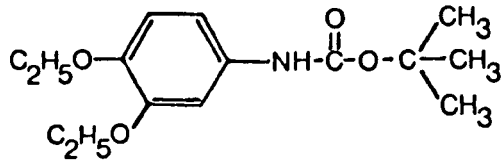
MDPC mol wt 220



10.0 : 2.20

45.5 : 10.0

S-32165 mol wt 281.3



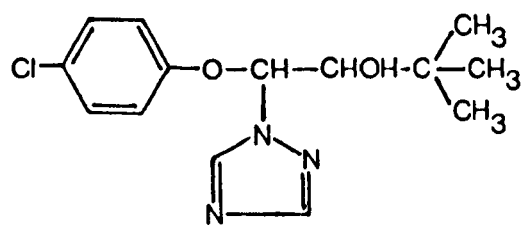
10.0 : 2.81

35.5 : 10.0

Ergosterol Biosynthesis Inhibitors

Triazoles

triadimenol mol wt 295.8

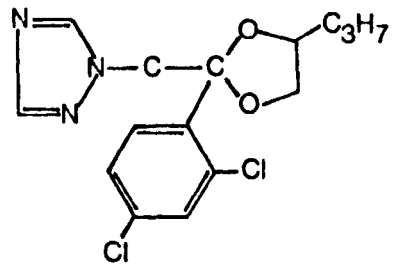


10.0 : 2.96

33.8 : 10.0

μM : $\mu g\ ml^{-1}$

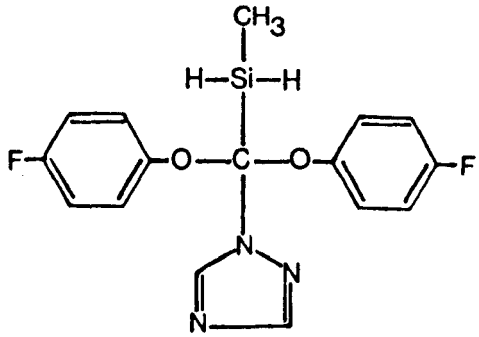
propiconazole mol wt 342.2



10.0 : 3.42

29.2 : 10.0

DPX H6573 mol wt 315.4

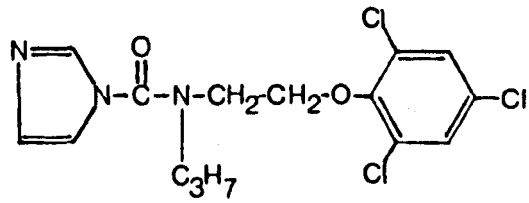


10.0 : 3.15

31.7 : 10.0

Imidazoles

prochloraz mol wt 376.7

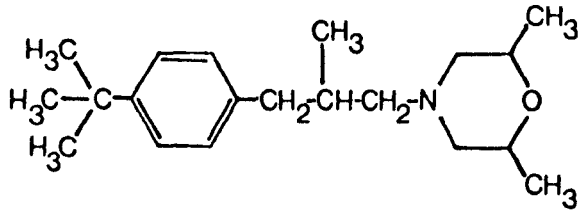


10.0 : 3.77

26.5 : 10.0

Morpholines

fenpropimorph mol wt 303.5



10.0 : 3.04

32.9 : 10.0

Appendix III Diagnostic media used for the identification of auxotrophic mutants. Most supplements prepared as 1 to 2% stock solutions and added to give a final concentration of 0.1 mg ml^{-1}. Vitamin-free casamino acids (Oxoid) was used at a final concentration of 1.25 mg ml^{-1}

Nutritional Supplement Symbols:

ADE	adenine	LEU	leucine
ALA	alanine	LYS	lysine
ARG	arginine	MET	methionine
ASN	asparagine	NIC	nicotinic acid
ASP	aspartate	ORN	ornitnine
BIO	biotin	PABA	p-aminobenzioc acid
CHO	choline	PANTO	pantothenic acid
CIT	citrulline	PDX	pyridoxin
CYS	cysteine	PHE	phenylalinine
CYTP	cytosine	PRO	proline
FOLATE	folic acid	RIBO	riboflavine
GLO	glutamic acid	SER	serine
GLY	glycine	THR	threonine
GUA	guanine	THY	thymine
HIS	histidine	TRP	tryptophan
HOMOCYS	homocysteine	TYR	tyrosine
ILE	isoleucine	URA	uracil
ILE	inositol	VAL	valine

Series A

PLATE NUMBER	1	2	3	4	5	6	7	8
1	ADE							
2	CYS	GUA						
3	TYR	CIT	THY					
4	GLU	RIBO	MET	URA				
5	PHE	CHO	PABA	ARG	CYTO			
6	FOLATE	ILE	VAL	NIC	LYS	ORN		
7	PRO	SER	POX	ASN	GLY	ALA	TRP	
8	BIO	ASP	HIS	THR	LEU	HOMOCYS	PANTO	INO
9	$(NH_4)_2SO_4$ + $Na_2S_2O_3$							
10	VITAMIN-FREE CASAMINO ACIDS							

Series B

PLATE NUMBER							
1	ARG	URA	PRO				
2	TYR	PHE	TRP				
3	VAL	LEU	ILE				
4	MET	THR	CYS				
5	SER	GLY	GLU	ALA			
6	HIS	LYS	ASP	ASN			
7	ADE	URA	THY				
8	BIO	CHO	INO	NIC	PABA	PANTO	RIBO

APPENDIX IV. UV-mutagenesis and selection of auxotrophic mutants in *P. herpotrichoides*: experimental conditions, isolation frequency and nutritional requirements of auxotrophs.

(SMYG = MYG supplemented with a range of vitamins, aminoacids and nucleic bases.

SMM = MM supplemented with casamino acids, vitamins, adenine and uracil. All supplements were used at the standard concentrations).

EXPERIMENT NUMBER	1								2		3	4	5	6	7		8	9			
STRAIN NUMBER	22-20								22-20		22-29	22-49	22-108	22-111	22-108		22-6	22-12			
PARENTAL MARKERS	BW-type								BW-type		ben-17	ben-21	hisA-1	argA-1	hisA-1		BWR-type	BWR-type			
UV DOSE ($Jm^{-2}s^{-1}$)	96								96		96	96	96	96	96		96	96			
SPORE CONCENTRATION (ml^{-1})	10^6								10^6		10^6	10^6	10^6	10^6	10^6		10^5	10^5			
SUSPENSION VOLUME (ml)	20								20		6	6	20	20	20		6	6			
INITIAL SPORE VIABILITY (%)	60.0								98.0		66.0	40.8	81.2	78.2	66.7		59.0	68.7			
RECOVERY MEDIUM	MYG					MYG			MYG		MYG	MYG	MYG	MYG	SMYG	SMM	MYG	SMYG	SMM	SMYG	SMM
FILTRATION ENRICHMENT	-	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
LIQUID HOLDING (24h)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
SURVIVAL OF VIABLE SPORES (%)	6.7	6.7	0.8	0.8	9.6	9.6	0.3	0.3	34.4	11.0	19.6	16.9	9.4	22.4	27.3	27.3	27.0	45.0	30.0	92.1	76.3
NUMBER OF COLONIES TESTED ON MM	112	256	96	256	432	256	-	240	624	1312	1040	1024	1136	1104	443	484	976	384	448	480	416
NUMBER OF AUXOTROPHS	1	1	1	3	4	1	-	0	5	1	6	6	7	1	2	4	0	5	1	1	0
FREQUENCY OF AUXOTROPHS (%)	0.9	0.4	1.0	1.2	0.9	0.4	-	<0.4	0.8	0.1	0.6	0.6	0.6	0.1	0.5	0.8	<0.1	1.3	0.2	0.2	<0.2
INDUCED AUXOTROPHIC REQUIREMENTS	adeA-1	tyr-1	lys-2	lysA-1 asnA-1 asnA-2	argA-1 adeA-2 asnB-4 ilvA-2	hisA-1			asnA-3 lys-3 adeB-3 lysC-6 metD-4	sA-1	sB-2 sB-3 metA-1 lysB-4 hisB-2 arg-2	sC-4 sC-5 adeC-4 adeA-5 hisC-3 trp-1	metB-2 metC-3 uraA-1 uraB-3 sC-6 ornC-5 argB-3	ilvA-1	argA-4 nic-1	leuA-1 leuA-2 serA-72 aux-4		nicA-2 nicA-3 nicA-4 nicA-6 s-71	nicA-5	lys-5	

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